CDK4/6 inhibition triggers anti-tumour immunity

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Cyclin-dependent kinases 4 and 6 (CDK4/6) are fundamental drivers of the cell cycle and are required for the initiation and progression of various malignancies1,2. Pharmacological inhibitors of CDK4/6 have shown significant activity against several solid tumours3–4. Their primary mechanism of action is thought to be the inhibition of phosphorylation of the retinoblastoma tumour suppressor, inducing G1 cell cycle arrest in tumour cells5. Here we use mouse models of breast carcinoma and other solid tumours to show that selective CDK4/6 inhibitors not only induce tumour cell cycle arrest, but also promote anti-tumour immunity. We confirm this phenomenon through transcriptomic analysis of serial biopsies from a clinical trial of CDK4/6 inhibitor treatment for breast cancer. The enhanced anti-tumour immune response has two underpinnings. First, CDK4/6 inhibitors activate tumour cell expression of endogenous retroviral elements, thus increasing intracellular levels of double-stranded RNA. This in turn stimulates production of type III interferons and hence enhances tumour antigen presentation. Second, CDK4/6 inhibitors markedly suppress the proliferation of regulatory T cells. Mechanistically, the effects of CDK4/6 inhibitors both on tumour cells and on regulatory T cells are associated with reduced activity of the E2F target, DNA methyltransferase 1. Ultimately, these events promote cytotoxic T-cell-mediated clearance of tumour cells, which is further enhanced by the addition of immune checkpoint blockade.

Our findings indicate that CDK4/6 inhibitors increase tumour immunogenicity and provide a rationale for new combination regimens comprising CDK4/6 inhibitors and immunotherapies as anti-cancer treatment.

We first assessed the impact of CDK4/6 inhibition in vivo using our recently described MMTV-rtTA/tet-O-HER2 transgenic mouse model of mammary carcinoma6. Cells derived from these tumours express the retinoblastoma (RB) tumour suppressor and arrest in response to CDK4/6 inhibition5. In three independent experiments, the CDK4/6 inhibitor abemaciclib caused G1 cell cycle arrest of bulky tumours, evidenced by an ~40% reduction in tumour volume at the 12-day end point (Fig. 1a). As expected, abemaciclib reduced tumour cell proliferation (Extended Data Fig. 1a). Expression analysis across a 3,826 cancer-related genes from MMTV-rtTA/tet-O-HER2 tumours (Fig. 1b) showed that abemaciclib downregulated genes within gene ontology (GO) and gene set enrichment analysis (GSEA) terms relating to cell cycle, mitosis, and E2F targets (Extended Data Fig. 1b–d). Strikingly, only two GO process terms were significantly enriched for genes upregulated by abemaciclib: ‘antigen processing and presentation of peptide antigen’ and ‘antigen processing and presentation’ (Fig. 1c). Specifically, genes encoding mouse major histocompatibility complex (MHC) class I molecules were upregulated in abemaciclib-treated tumours (H2d1, H2k1, and B2m), as were genes directing peptide cleavage (Erap1), peptide transporters (Tap1 and Tap2), and transporter–MHC interactions (Tapbp) (Fig. 1d). Moreover, abemaciclib treatment in vitro increased expression of the orthologous genes in human breast cancer cell lines (MDA-MB-453, MCF7, and MDA-MB-361) (Fig. 1e and Extended Data Fig. 2a) and palbociclib, another CDK4/6 inhibitor, yielded similar results (Extended Data Fig. 2b). Importantly, treatment with either agent increased cell-surface expression of HLA-MHC class I proteins (Extended Data Fig. 2c). The CDK4/6 inhibitor-induced increase in expression of antigen processing and presentation genes was also observed in a patient-derived breast cancer xenograft of a treatment-refractory breast cancer (PDX 14-07, previously described6) (Fig. 1f). Furthermore, analysis of The Cancer Genome Atlas (TCGA) data5 revealed that breast cancers harbouring CCND1 amplification (that is, enhanced CDK4/6 activity) display significantly lower expression of HLA-A, HLA-B, and HLA-C than non-amplified tumours (Extended Data Fig. 2d).

To determine the functional consequences of increased antigen presentation gene expression, we treated ovalbumin (OVA)-expressing mouse cancer cell lines (MMTV-PyMT-S2WT3-OVA and B16-OVA) with abemaciclib. Abemaciclib increased expression of MHC class I-bound SIINFEKL, an eight-aminoc-acid peptide derived from OVA (Extended Data Fig. 2e), confirming enhanced antigen processing and presentation. Co-culture of abemaciclib-pretreated tumour cells with MHC-class-I-restricted OVA-specific CD8+ T cells (OT-I cells) significantly increased OT-I cell proliferation, interferon-γ (IFN-γ) production, and tumour-necrosis factor-α (TNF-α) production (Fig. 1g and Extended Data Fig. 2f). CD8+ T-cell proliferation was both antigen-specific (P14 CD8+ T cells, which recognize an irrelevant antigen, did not increase their proliferation) and MHC-class-I-specific (MHC class I blocking antibody partly suppressed OT-I T-cell proliferation) (Fig. 1g and Extended Data Fig. 2f).

In agreement with other reports5,6, abemaciclib did not directly induce breast cancer cell apoptosis; rather, it caused cell cycle arrest and induced cellular phenotypes consistent with senescence (Extended Data Fig. 3a, b). Cellular senescence has been associated with apoptosis resistance6; indeed, abemaciclib reduced tumour cell poly(ADP-ribose) polymerase cleavage and suppressed the apoptotic response to staurosporine (Extended Data Fig. 3c, d). Collectively, these results establish that CDK4/6 inhibitors induce breast cancer cell cytostasis without directly causing their apoptosis, and enhance their capacity to present antigen and stimulate cytotoxic T cells.
We next sought mechanistic insight for the increase in antigen presentation after CDK4/6 inhibition. Genome-wide transcriptomic analysis of three human breast cancer cell lines and PDX 14-07 tumours revealed that abemaciclib upregulated expression of genes within GO terms related to IFN signalling and cellular defence response to virus (Fig. 2a, b and Extended Data Fig. 4a). Several IFN-sensitive transcription factors (for example, STAT1, STAT2, IRF2, IRF6, IRF9, and NLRc5) were upregulated in all three cell lines, PDX 14-07 tumours, and MMTV-PyMT-S2WTP3 cells (Fig. 2c, d and Extended Data Fig. 4b, c). Similar trends were observed when abemaciclib was given in conjunction with anti-oestrogen therapy—the current standard in luminal breast cancer treatment (Extended Data Fig. 4d). Expression of other IFN-stimulated genes (ISGs)—OAS1, OAS2, IFIT1, IFIT2, BST2, SP100, RSAD2—was also enhanced in cell lines and PDX tumours, suggesting global upregulation of an IFN-driven transcriptional program (Extended Data Fig. 4e, f). Consistent with active IFN signalling, both phosphorylated and total STAT1 protein were increased after abemaciclib treatment (Extended Data Fig. 4g). Furthermore, forced overexpression of the endogenous CDK4/6 inhibitor CDKNa2 (encoding P16INK4a) increased expression of STAT1, B2M, and MHC class I genes (Extended Data Fig. 4h), suggesting that these are ‘on-target’ effects.

Importantly, we made similar observations in MMTV-rtTA/tetO-HER2 tumour tissues after abemaciclib treatment. Enriched GSEA expression signatures included ‘allograft rejection’, ‘interferon alpha response’, and ‘interferon gamma response’ (Fig. 2e). Specifically, abemaciclib also significantly increased tumour expression of IFN-responsive transcription factors in these tumours (Fig. 2f), as well as that of IFN-inducible T-cell chemottractants and other ISGs involved in lymphocyte adhesion and co-stimulation (Extended Data Fig. 4i, j). STAT1 protein levels were also higher within tumour cells of the abemaciclib-treated cohort (Fig. 2g). In keeping with their roles in regulating antigen presentation via MHC class I, in vivo expression of transcription factors Stat1 and Nlr5c5 was tightly correlated with that of H2d1, H2k1, Tap1, Tap2, and Tapbp in MMTV-rtTA/tetO-HER2 tumours (Extended Data Fig. 4k). Thus, CDK4/6 inhibition directly induces ISG expression in tumour cells, probably explaining their enhanced capacity for antigen presentation.

We next interrogated genome-wide expression data from the NeoPaAlna trial, in which patients with primary oestrogen-receptor-positive breast cancer underwent tumour biopsies before starting palbociclib and serially after 2 and 12 weeks of treatment (Extended Data Fig. 5a). As expected, palbociclib treatment significantly downregulated genes within the ‘E2F target’ and other cell-cycle-related signatures (Extended Data Fig. 5b). When we compared upregulated genes from biopsies taken before initiation of palbociclib with those after 12 weeks of treatment, three of the top five ranked ‘Hallmarks’ GSEA signatures were ‘allograft rejection’, ‘inflammatory response’, and ‘interferon gamma response’ (Fig. 2h), and these gene sets were already significantly upregulated after 2 weeks of palbociclib treatment (Extended Data Fig. 5c). These signatures are similar to those we observed to be upregulated in the tumours from abemaciclib-treated MMTV-rtTA/tetO-HER2 mice (Fig. 2e), and demonstrate the clinical relevance of our findings.

We speculated that heightened ISG expression after CDK4/6 inhibition might be caused by enhanced production of tumour cell IFN. The Janus kinases (JAKs) mediate intracellular signalling after ligand-dependent activation of IFN receptor, and the JAK inhibitor ruxolitinib completely mitigated abemaciclib-induced increases in tumour cell p-STAT1 and total STAT1 (Extended Data Fig. 6a), suggesting that CDK4/6 inhibition stimulates production of tumour cell IFN. Notably, enzyme-linked immunosorbent assay (ELISA) did not detect IFN-α, -β, or -γ in conditioned medium of abemaciclib-treated tumour cells (not shown), and neutralizing antibodies against IFN-α and IFN-γ did not mitigate IFN signalling (Extended Data Fig. 6b–d). However, gene expression and protein secretion of type III IFNs (interleukin (IL)-29, IL-28a, and IL-28b) were significantly increased in abemaciclib-treated tumour cells (Fig. 3a and Extended Data Fig. 6e, f). Neutralizing antibodies to type III IFNs reversed abemaciclib-induced increases in tumour cell function (Extended Data Fig. 6f). These data suggest that CDK4/6 inhibition increases tumour cell production of type III IFNs to drive ISG expression in an autocrine fashion.

Increased tumour cell IFN signalling via type III IFN production has previously been shown to occur in response to DNA demethylation caused by agents such as 5-aza-cytidine, which inhibits DNA methyltransferases (DNMTs)10. In that context, DNMT inhibition reduces methylation of endogenous retroviral genes (ERVs), triggering ‘viral mimicry’ and a double-stranded RNA (dsRNA) response, in turn stimulating type III IFN production to activate ISGs10. Notably, the mammalian DNA methyltransferase gene DNMT1 is a bona fide E2F target gene, and CDK4/6 enzyme activity can enhance DNMT1 gene

Figure 1 | CDK4/6 inhibitors induce tumour regression and increase antigen presentation. a, Impact of abemaciclib treatment on MMTV-rtTA/tetO-HER2 tumour volume (two-way analysis of variance (ANOVA), vehicle, n = 17; abemaciclib, n = 22 tumours). b–d, In vivo experimental schema depicted in b (vehicle, n = 11; abemaciclib, n = 12 tumours). GO terms with P < 0.05 (c) and expression of antigen presentation genes (d) are shown. e, f, Antigen presentation gene expression in cells (e) (7 days; n = 3) and PDX tumours (f) (21–28 days; vehicle, n = 4; abemaciclib, n = 2 tumours) after abemaciclib treatment. DMSO, dimethylsulfoxide.

Figure 2 | CDK4/6 inhibitors induce tumour regression and increase antigen presentation. a, Impact of abemaciclib treatment on MMTV-rtTA/tetO-HER2 tumour volume (two-way analysis of variance (ANOVA), vehicle, n = 17; abemaciclib, n = 22 tumours). b–d, In vivo experimental schema depicted in b (vehicle, n = 11; abemaciclib, n = 12 tumours). GO terms with P < 0.05 (c) and expression of antigen presentation genes (d) are shown. e, f, Antigen presentation gene expression in cells (e) (7 days; n = 3) and PDX tumours (f) (21–28 days; vehicle, n = 4; abemaciclib, n = 2 tumours) after abemaciclib treatment. DMSO, dimethylsulfoxide.

Figure 3 | CDK4/6 inhibitors induce tumour regression and increase antigen presentation. a, Impact of abemaciclib treatment on MMTV-rtTA/tetO-HER2 tumour volume (two-way analysis of variance (ANOVA), vehicle, n = 17; abemaciclib, n = 22 tumours). b–d, In vivo experimental schema depicted in b (vehicle, n = 11; abemaciclib, n = 12 tumours). GO terms with P < 0.05 (c) and expression of antigen presentation genes (d) are shown. e, f, Antigen presentation gene expression in cells (e) (7 days; n = 3) and PDX tumours (f) (21–28 days; vehicle, n = 4; abemaciclib, n = 2 tumours) after abemaciclib treatment. DMSO, dimethylsulfoxide.
CDK4/6 inhibition stimulates IFN signalling. a, b, Top-ranked GO terms in abemaciclib-treated tumour cells (a) (7 days; n = 3) or PDX tumours (b) (21–28 days; vehicle, n = 4; abemaciclib, n = 2 tumours). c, d, Interferon-responsive gene expression from samples in a and b. e, f, Upregulated GO terms (e) and expression of IFN-α-responsive transcription factors (f) in abemaciclib-treated MMTV-rTATetO-HER2 tumours (12 days; vehicle, n = 11; abemaciclib, n = 12 tumours). ES, enrichment score; NES, normalized enrichment score.

Our observation that CDK4/6 inhibition enhances the efficiency of tumour cell antigen presentation prompted us to examine the tumour immune microenvironment. Flow cytometric analysis of MMTV-rTATetO-HER2 tumours revealed that abemaciclib did not alter fractions of most types of tumour-infiltrating leukocytes (Extended Data Fig. 9a). Strikingly, however, there were significant increases in CD3+ T cells (Fig. 4a) and reductions in CD4+ FOXP3+ regulatory T cells.

Figure 3 | CDK4/6 inhibitors suppress DNMT1, inducing viral mimicry. a, Tumour cell type III IFN secretion measured by ELISA (7 days). b, Impact of type III IFN neutralization on phospho- and total STAT1 protein (7 days). c, d, DNMT1 expression after abemaciclib or control in cells (b) (7 days; n = 3) or PDX tumours (d) (21–28 days; vehicle, n = 4; abemaciclib, n = 2 tumours). e–f, ERV3-1 expression in samples from c and d. g, dsRNA levels in abemaciclib-treated tumour cells (7 days; n = 2).

Figure 2 | CDK4/6 inhibition stimulates IFN signalling. a, b, Top-ranked GO terms in abemaciclib-treated tumour cells (a) (7 days; n = 3) or PDX tumours (b) (21–28 days; vehicle, n = 4; abemaciclib, n = 2 tumours). c, d, Interferon-responsive gene expression from samples in a and b. e, f, Upregulated GO terms (e) and expression of IFN-α-responsive transcription factors (f) in abemaciclib-treated MMTV-rTATetO-HER2 tumours (12 days; vehicle, n = 11; abemaciclib, n = 12 tumours). ES, enrichment score; NES, normalized enrichment score.

expression in a RB–E2F-dependent fashion. Strikingly, tumour cell levels of DNMT1 (but not DNMT3A) mRNA and protein fell markedly and rapidly in abemaciclib-treated cells (Fig. 3c and Extended Data Fig. 7a–c). Similarly, abemaciclib reduced DNMT1 mRNA levels in vivo in both PDX 14-07 and MMTV-rTATetO-HER2 tumours (Fig. 3d and Extended Data Fig. 7d). These were on-target effects, as abemaciclib did not reduce DNMT1 levels in tumour cells expressing short hairpin RNA (shRNA) targeting RB1 (Extended Data Fig. 7e). The abemaciclib-related increases in IFNLR2, STAT1, and HLA-B expression were also mitigated in shRNA-RB1 cells (Extended Data Fig. 7e), supporting the concept that the RB–E2F–DNMT1 axis mediates type III IFN production and its downstream consequences.

Consistent with previous studies, suppression of DNMT1 was associated with reduced DNA methylation at ERV sequences: combined bisulfite restriction analysis showed that abemaciclib decreased tumour cell DNA methylation at the 5’ long terminal repeat (LTR) of ERV3-1 (Extended Data Fig. 7f). Accordingly, expression of certain ERVs was increased in vitro and in vivo (Fig. 3e, f and Extended Data Fig. 7g), accompanied by significantly higher levels of dsRNA within tumour cells (Fig. 3g and Extended Data Fig. 7h). In turn, expression of the dsRNA pattern recognition receptors RIG-1 (DDX58), LGP2 (DHX58), and MDA5 (IFIH1) was significantly increased in cell lines and PDX tumours (Extended Data Fig. 7i, j). In further support of abemaciclib-mediated DNMT1 suppression as the trigger for this cascade of events, overexpression of DNMT1 in tumour cells attenuated the abemaciclib-induced increase in expression of dsRNA response genes, type III IFN, and ISGs (Fig. 3h and Extended Data Fig. 7k).

Independent of altered ERV expression, CDK4/6 inhibitors could theoretically increase tumour cell immunogenicity by inducing a ‘senescence-associated secretory phenotype’ (SASP). Although abemaciclib increased tumour cell β-galactosidase activity (Extended Data Figs 3b and 8a), expression of key SASP factors was not increased (Extended Data Fig. 8b, c and Supplementary Table 1). In contrast, doxorubicin increased tumour cell expression of SASP genes (Extended Data Fig. 8d), consistent with the notion that the SASP is more pertinent to DNA damage response-associated senescence and does not explain tumour immunogenicity after CDK4/6 inhibition.
in vitro, cfse, carboxyfluorescein succinimidyl ester. f, Ki-67/FoxP3 staining of MMTV-rtTA/tetO-HER2 tumours (12 days; scale bar, 50 μm, n = 7). g, MMTV-rtTA/tetO-HER2 tumour volume after treatment with abemaciclib with or without anti-PD-L1. h, Unpaired two-tailed t-tests (a–c, f), one-way ANOVA corrected for multiple comparisons (d, j), two-way ANOVA (g) corrected for multiple comparisons (e, h). Error bars, s.d., except (g, j), s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. For source data, see Supplementary Table 2.

Figure 4 | CDK4/6 inhibition modulates the immune milieu. a–c, Intratumoral T cells (a), TReg cells (b), and TReg:CD8 ratios (c) in MMTV-rtTA/tetO-HER2 tumours (12 days of treatment; vehicle, n = 15; abemaciclib, n = 17 tumours). d, TReg cells in spleens of tumour-free mice (12 days; vehicle and palbociclib, n = 8; abemaciclib, n = 7 mice). e, T-cell proliferation in vitro. cfse, carboxyfluorescein succinimidyl ester. f, Ki-67/FoxP3 staining of MMTV-rtTA/tetO-HER2 tumours (12 days; scale bar, 50 μm, n = 7). g, MMTV-rtTA/tetO-HER2 tumour volume after treatment with abemaciclib with or without anti-PD-L1. h, Unpaired two-tailed t-tests (a–c, f), one-way ANOVA corrected for multiple comparisons (d, j), two-way ANOVA (g) corrected for multiple comparisons (e, h). Error bars, s.d., except (g, j), s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. For source data, see Supplementary Table 2.

In tumour-free mice, both abemaciclib and palbociclib significantly reduced TReg numbers and the TReg:CD8 ratio in the spleen and lymph nodes, demonstrating tumour-independent effects of these agents (Fig. 4d and Extended Data Fig. 9e, f). Plasma levels of anti-nuclear and anti-dsDNA antibodies were unchanged (data not shown; Extended Data Fig. 9g), indicating no significant accompanying autoimmune phenotype. To understand why TReg cells were reduced in number, we analysed the impact of CDK4/6 inhibitors on various aspects of TReg biology. Palbociclib and abemaciclib each significantly reduced total thymic mass, decreased immature CD4+CD8+ double-positive thymocytes, and increased the fractions of CD4+ and CD8+ single-positive thymocytes (Extended Data Fig. 9h–k), consistent with previous reports of CDK6 inhibition14. However, proportions of thymic TReg cells were unchanged, arguing against a defect in thymus-derived TReg production (Extended Data Fig. 9l). Similarly, CDK4/6 inhibitors did not prevent in vitro differentiation of naive CD4+ T cells into TReg cells, and did not affect rates of TReg apoptosis (Extended Data Fig. 9m, n). We then turned our attention to TReg proliferation. In vitro, abemaciclib markedly reduced the proliferation of CD4+CD25+ TReg cells isolated from spleens and lymph nodes of wild-type mice, but had a weaker effect on the proliferation of CD4+CD25+ and CD8+ T cells (Fig. 4e). Likewise, the fraction of proliferating TReg cells, but not CD8+ T cells, within abemaciclib-treated tumour tissue was significantly reduced (Fig. 4f and Extended Data Fig. 9o). Dnmt1 inhibition in TReg cells has previously been reported to suppress their proliferation, ostensibly via hypomethylation of the Cdkn1a promoter, thus increasing expression of the Cdkn1a protein p21—a potent and promiscuous cell cycle inhibitor15. In tumour-free mice treated with abemaciclib for 12 days, Dnmt1 expression was reduced in TReg cells by ∼70%; in contrast, Dnmt1 mRNA was unchanged in naive CD4+ T cells and fell by only 20% in CD8+ T cells (Extended Data Fig. 9p). Mirroring the magnitude of these changes, expression of Cdkn1a increased 75-fold in TReg cells, 2.8-fold in CD8+ T cells, and was unchanged in naive CD4+ T cells (Extended Data Fig. 9q). Therefore, the relatively selective suppression of TReg proliferation after CDK4/6 inhibition could relate to enhanced cell cycle inhibition due to overexpression of p21, associated with reduced levels of Dnmt1.

Tumour cells presenting antigen via MHC class I can be recognized by cytotoxic T lymphocytes (CTLs), and TReg cells suppress CTL responses, in part by promoting their exhaustion16. Therefore, to determine whether CTLs were involved in abemaciclib-mediated therapeutic efficacy, we implanted MMTV-rtTA/tetO-HER2 tumour fragments orthotopically into athymic Foxn1nu mice maintained on doxycycline, and treated them once tumours were established. In contrast to tumours in immunocompetent mice, abemaciclib-treated tumours in nude mice continued to grow, albeit at a significantly slower rate than vehicle-treated tumours, and in no case did tumours regress (Extended Data Fig. 10a, b). We therefore treated MMTV-rtTA/tetO-HER2 tumour-bearing mice with an anti-CD8 antibody before abemaciclib administration. Abemaciclib-induced tumour regression, which was observed in control IgG-treated mice, was significantly mitigated by CD8 neutralization (Fig. 4g and Extended Data Fig. 10c).
Hence, tumour regression mediated by CDK4/6 inhibition is, at least in part, dependent on the presence of CTLs.

Intratumoural CD8+ T cells in abemaciclib-treated mice displayed markedly reduced expression of PD-1, Tim-3, CTLA-4, and LAG3 (Fig. 4h and Extended Data Fig. 10d–g), markers typically indicative of T-cell exhaustion17. The number of inhibitory receptors detected on any given CTL was lower in abemaciclib-treated MMTV-rtTA/tetO-HER2 and MMTV-PyMT tumours (Fig. 4i and Extended Data Fig. 10h). Specifically, the fractions of both PD-1high and PD-1+/ Tim-3+ CTLs were significantly reduced (Fig. 4h and Extended Data Fig. 10d). Levels of Ifgy mRNA (the main effector cytokine of CTLs) were more than fourfold higher in abemaciclib-treated bulk tumour tissues (Extended Data Fig. 10i). Collectively, these results indicate that CDK4/6 inhibition results in activation of CTLs, which are necessary for abemaciclib-induced tumour regression.

Increased tumour cell antigen presentation coupled with anti-tumour T-cell responses suggested that immune checkpoint blockade might further enhance the efficacy of abemaciclib. We treated tumour-bearing mice with either vehicle or abemaciclib, which might further enhance the efficacy of abemaciclib. We treated tumour-bearing mice with either vehicle or abemaciclib, and a control IgG or an anti-PDL1 antibody (Extended Data Fig. 10j). In agreement with our initial experiments, abemaciclib-treated tumours initially decreased in volume before stabilizing and ultimately resuming growth by day 21 (Fig. 4j). In contrast, tumours in mice treated with abemaciclib and anti-PDL1 combination therapy regressed to the greatest degree (~70% reduction in tumour volume by day 13), and did not resume growth by day 35 (Fig. 4j and Extended Data Fig. 10k). Similar results were obtained for the CT-26 colorectal carcinoma model, wherein combination therapy induced complete tumour regression in all cases, and rendered mice disease-resistant when re-challenged with a subsequent injection of CT-26 tumour cells 5 weeks after stopping therapy (Extended Data Fig. 10l, m).

Collectively, our data in cell lines, animal models, and patients with breast cancer demonstrate that CDK4/6 inhibitors enhance anti-tumour immunity by overcoming two central mechanisms of tumour immune evasion (Extended Data Fig. 10n). First, they increase the functional capacity of tumour cells to present antigen. Second, they reduce the immunosuppressive Treg population by suppressing their proliferation. Intriguingly, these phenomena might not only cooperate to enhance tumour immunogenicity, but could also have the same mechanistic underpinning: suppression of the RB–E2F axis, leading to reduced DNMT1 expression and thus hypomethylation of genes that regulate immune function10,12,15. The selective suppression of Treg proliferation (and not that of CD8+ or conventional CD4+ T cells) might relate to the fact that Treg cells express higher levels of Rb1, a key mediator of CDK4/6 pathway modulation16 (for example, Rb1 expression is 3.1-fold higher in Treg cells than in CD8+ T cells19). With respect to breast cancer specifically, we note that most human breast cancers are oestrogen-receptor positive, and these tumours are considered inherently less immunogenic. Interestingly, they generally retain retinoblastoma tumour suppressor function and, furthermore, a high number of Treg cells in these tumours predicts a poor clinical outcome20. Our findings therefore suggest that CDK4/6 inhibitors might enhance the susceptibility of such tumours to immune checkpoint blockade.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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METHODS

Animal experiments. MMTV-ERT²/tetO-HER2 mice were maintained in house. Female FVB MMTV-PyMT, Balb/c (6–7 weeks old) and Foxn1nu (8 weeks old) mice were purchased from The Jackson Laboratory. Female FVB mice (7 weeks old) were purchased from Taconic Biosciences. P14-TCR transgenic mice (a gift from K. Wucherpfennig) and OT-1 mice (C57BL/6 Tg[TcraTcrb]1100Mjb/J; The Jackson Laboratory) were used as a source of CD8+ T cells for in vitro co-culture assays. FVB CD45.2+ mice (a gift from D. Tenen) were used as a source of T cells for in vitro drug assays.

Tumours were induced and maintained in MMTV-ERT²/tetO-HER2 mice using doxycycline as previously described. For experiments in transgenic MMTV-ERT²/tetO-HER2 and MMTV-PyMT mice, tumours measured 4–10 mm at treatment initiation. Before treatment, mice were assigned into groups of equal average tumour volume. For CT-26 experiments, 1 × 10^6 tumour cells were injected subcutaneously in 1:1 PBS:Matrigel into Balb/c mice, and treatment was initiated 1 week later. For re-challenge experiments, 5 × 10^6 tumour cells were injected subcutaneously in 1:1 PBS:Matrigel 33 days after treatment was stopped. PDX 14-07 was established from a biopsy of a liver metastasis from a patient with ER+/HER2+ breast cancer as previously described.

For studies in Foxn1nu mice, MMTV-ERT²/tetO-HER2 tumour fragments were orthotopically implanted bilaterally into nude mice, as previously described. Before treatment initiation, mice were randomized into groups of equal average tumour volume (5–10 mm diameter). For in vivo experiments, samples sizes were determined on the basis of knowledge of inter-tumour growth rate variability, gained from previous model-specific experience. Tumour-bearing mice were randomly assigned to treatment groups, ensuring that baseline tumour volumes were balanced between treatment arms. Tumours were measured by calliper two or three times per week, and the volume was calculated as previously described. For tumour growth kinetic analysis, we performed two-way ANOVA or one-way ANOVA tests with correction for multiple comparisons using Sidák’s multiple comparisons test. No animals were excluded from analyses. Ex vivo analyses of tissue samples were carried out in a blinded fashion such that the investigators were unaware of treatment groups. Source data for tumour volume measurements can be found in Supplementary Table 2.

Abemaciclib (75–90 mg per kg (body weight), as noted for individual experiments, Haoyuan Chemexpress, prepared as previously described) and palbociclib (90 mg per kg, Haoyuan Chemexpress, diluted in 50 nM sodium d-lactate) were administered daily by oral gavage. For CD8 depletion experiments, mice were injected intraperitoneally with either α-CD8 antibody (400 µg; clone YTS 169.4, BioXcell) or isotype control (400 µg; clone LTF-2, BioXcell) 48 and 24 h before beginning abemaciclib therapy (90 mg per kg) and every 4 days thereafter. For combination therapy, treatment with abemaciclib was initiated at a dose of 90 mg per kg daily for 3 days; thereafter, the dose was dropped to 75 mg per kg daily and intra-peritoneal injection of either α-PDL1 antibody (200 µg every 72 h; BioXcell, clone 10E9G2) or isotype control antibody (200 µg every 72 h, clone LTF-2, BioXcell) was added to the regimen.

Mice were euthanized using CO₂ inhalation. All mouse experiments were performed in accordance with federal laws and institutional guidelines as approved by the Institutional Animal Care and Use Committees of Dana-Farber Cancer Institute, Harvard Medical School, and Boston Children’s Hospital. The maximum tumour diameter permitted under the relevant animal protocols is 25 mm, and this limit was not exceeded in any experiment. Immunohistochemistry and immunofluorescence. Immunostaining for Ki-67 (Vector), HER2 (AB16901; Abcam), and STAT1 (AB47425; Abcam) was performed using NIS Elements software, and five to ten fields were analysed per tumour. Immunofluorescence for FoxP3 (clone FJK-16s; eBioscience), CD8 (clone 4S5M15; eBioscience), and Ki-67 (clone SP6; Thermo Scientific) was performed as previously described21. Secondary antibodies (AF488 AffiniPure donkey anti-mouse IgG, Cy3 AffiniPure donkey anti-rabbit IgG) were from Jackson ImmunoResearch Laboratories. Images were acquired with a Yokogawa spinning-disc confocal microscope. For tumours, the fold-change in normalized read count was determined for each gene. For these genes, the fold-change in normalized read count was determined for each replicate experiment, and the mean fold change was calculated for each gene. Genes with a fold change of >2 (DMSO versus abemaciclib) were then included for analysis using Gene Ontology enrichment analysis. Transcriptome methodology. AmpliSeq libraries were constructed and sequenced on the Ion Torrent Proton platform (Thermo Fisher) according to the manufacturer’s instructions as previously described. For human gene analysis in cell lines, the Ion AmpliSeq Transcriptome Human Gene Expression Kit is designed for targeted amplification of over 20,000 human RefSeq genes simultaneously in a single primer pool. A short amplicon (~110 base pairs (bp)) is amplified for each target gene. Since an AmpliSeq transcriptome mouse kit is not commercially available, an Ion AmpliSeq Custom Panel was designed by the manufacturer (Thermo Fisher) using Ion AmpliSeq Designer for targeted amplification of 3,826 mouse genes that were most relevant for our studies (one short amplicon for each gene) in one primer pool for mouse studies. For each sample, 10 ng of total RNA was used for cDNA library preparation. Multiple libraries were multiplexed and clonally amplified using the Ion OneTouch 2 System (Thermo Fisher), and then sequenced on an Ion Torrent Proton machine (Thermo Fisher). Data were first analysed by Torrent Suite and ampliSeqRNA analysis plugin (Thermo Fisher) to generate count data.

Mouse transcriptome analysis. Raw read counts per gene were mapped to corresponding human homologues, using homology information from the Mouse Genome Informatics database (The Jackson Laboratory). For GO analysis, differential gene expression was determined using DESeq2 (ref. 26) and genes were ranked according to log2(fold change) (maximum a posteriori). For GSEA analysis, we used the GSEAPreranked tool, with the above-ranked gene list, using MSigDB v5.1 Hallmarks gene sets collection and the ‘classic’ method for calculating enrichment scores27,28.

Human transcriptome analysis. Analyses were performed on normalized read counts per gene for each GO. For GSEA analysis, only genes with an absolute read count of >100 were included. For these genes, the fold-change in normalized read count was determined for each replicate experiment, and the mean fold change was calculated for each gene. Genes with a mean fold change of >2 (DMSO versus abemaciclib) were then included for analysis using Gene Ontology enrichment analysis.

TCGA analysis. Gene expression data were obtained using the cBioPortal for Cancer Genomics (http://www.cbioportal.org). Data were obtained from the breast cancer data set ‘TCGA, Provisional (1105 samples)’ for comparisons between cyclin D1 Shanks deletions, amplified, gain, and diploid tumours.

Reverse transcription quantitative PCR. Reverse transcription quantitative PCR (RT-qPCR) performed as previously described. Primer sequences used for qPCR were as follows. Ifng (mouse) forward: 5’-ATG-AAC-GCT-ACA-ACA-TGC-ATC-3’; reverse: 5’-CCA-TCC-TTT-TGG-CAG-TTC-CTC-3’. Tap1 (mouse) forward: 5’-GGA-GTC-GTG-GTG-GTG-GGA-GG-3’; reverse: 5’-CCT-GGC-ACA-TAA-CTG-ATG-CTG-3’. 18S (mouse) forward: 5’-CGG-GTG-GTG-GTG-GTG-GGA-GG-3’; reverse: 5’-GGG-CCT-GGC-ACA-TAA-CTG-ATG-CTG-3’.
ATG-GCT-CTA-CTT-3′; reverse: 5′-CTC-CCA-CTT-TGA-GCA-GTC-CC-3′. TqCp (mouse) forward: 5′-GGC-CTG-TCT-AAG-CAA-GCT-GGC-3′; reverse: 5′-CCA-TCT-TGA-ACT-GTA-GCT-GTG-G-3′. Erpal (mouse) forward: 5′-TAA-TTG-AGA-CTC-ATT-CCC-TTG-CA-3′; reverse: 5′-AAA-ATC-GTA-AGG-ATA-GTG-AGG-TT-G-3′. \(\text{Plk}\) (mouse) forward: 5′-TGG-GAC-ATC-GAG-GAT-ATG-G-3′; reverse: 5′-CTT-TGT-GCT-ATG-CCA-GCA-AAC-3′. \(\text{Tdt}\) (mouse) forward: 5′-TGG-GAC-ATC-GAG-GAT-ATG-G-3′; reverse: 5′-CTT-TGT-GCT-ATG-CCA-GCA-AAC-3′.

**Tumour cell/OT-I co-culture assay.** MMTV-PyMT-S2WTP3-OVA cells were pretreated with abemaciclib (500 nM or 1,000 nM, respectively) for 7 days. Tumour cells were isolated from spleens and lymph nodes of OT-1 or P14 mice using a CD8\(^+\) T-cell isolation kit (Miltenyi Biotec) with an autoMACS Pro Separator. Isolated T cells were suspended in RPMI (ATCC) with 5% FBS, labelled with 5\(\mu\)M CFSE (Life Technologies) for 10 min in the dark at room temperature, and washed twice in 10× volume of T-cell media (RPMI, 10% FBS, 55\(\mu\)M 2-mercaptoethanol (Gibco)). One hundred thousand CD8\(^+\) T cells were co-cultured with abecarnil- or control-pretreated tumour cells at a ratio of 1:8 tumour cells:T cells (MMTV-PyMT-S2WTP3-OVA) or 1:4 tumour cells:T cells (B16-OVA) in a final concentration of 10\(\mu\)g ml\(^{-1}\) of the appropriate antibody with 2.5 ng ml\(^{-1}\) IL-7 (Peprotech), 50 ng ml\(^{-1}\) IL-15 (Peprotech), and 2 ng ml\(^{-1}\) IL-2 (Peprotech) for 48 h (MMTV-PyMT-S2WTP3-OVA) or 72 h (B16-OVA) at 37°C in the dark. At the experimental end point, cytokines in conditioned media were analysed by ELISA, and CD\(8^+\) T-cell proliferation was determined by CFSE-dilution by flow cytometry.

**Overexpression vectors and transfection.** MMTV-PyMT-S2WTP3 cells were infected with lentiviral particles expressing the pHAGE-deltaOα-nuclear green plasmid as previously described, and infected cells were isolated by fluorescence-activated cell sorting.

MDA-MB-453 and BT474 cells were transiently transfected with pBabeuro-3-p16Flag (Addgene catalogue number 24934) using Lipofectamine 3000 (Thermo Fisher) according to the manufacturer's instructions. Seventy-two hours after transfection, cells were selected in puromycin for 48 h. Overexpression of p16 was confirmed by anti-Flag western blots.

For overexpression of DNMT1, MDA-MB-453 cells were transfected with the pcDNA3/Myc-DNMT1 vector (Addgene catalogue number 36939) using Lipofectamine 3000 according to the manufacturer's instructions. Cells were treated with abecarnil 72 h after transfection.

**ELISAs.** Cells were treated with DMSO or abecarnil (500 nM) for 7 days. For the last 24 h, culture media were replaced with serum-free media. After concentration of conditioned medium using Amicon Ultra centrifugal filters (Millipore), cytokines were analysed according to the manufacturer's recommendations: human IFN gamma ELISA Ready-SET-Go! (Affymetrix ebioscience), human TNF alpha ELISA Ready-SET-Go! (Affymetrix ebioscience), VeriKine human IFN alpha ELISA kit (PBL assay science), human IL-28B quantikine ELISA kit (R&D Systems), human IL-28A DuoSet ELISA (R&D Systems), and human IL-29 DuoSet ELISA (R&D Systems). Cytokine production by OT-I T cells in OVA/OT-1 co-cultures was measured by mouse IFN gamma ELISA Ready-SET-Go! (Affymetrix ebioscience) and mouse TNF alpha ProbeLink ELISA Kit (R&D Systems) according to the manufacturer's instructions. Absorbance was measured on a Synergy Neo plate reader (BioTek) using Gen5 software.

**IFN neutralization experiments.** Cell lines were treated with DMSO or abecarnil (500 nM) for 7 days. For the duration of drug treatment, neutralizing antibodies were applied including IFN-\(\gamma\) (1\(\mu\)g ml\(^{-1}\), R&D Systems, MAB2851), IFN-\(\alpha\) (2.0–2.5\(\mu\)g ml\(^{-1}\), R&D Systems, 21100–1), IL-28B (0.5\(\mu\)g ml\(^{-1}\), R&D Systems, MAB1587), or IL-12 (1–1.9\(\mu\)g ml\(^{-1}\), R&D Systems, MAB15981–100). Successful neutralization was validated using recombinant human IFN-\(\gamma\) (Peprotech, 250 pg ml\(^{-1}\)) and IFN-\(\alpha\) (Life Technologies, 250 pg ml\(^{-1}\)). Recombinant proteins were administered 24 h before protein collection.

**Analysis of methylation of the ER\(\alpha\) 3′-UTR.** Genomic DNA was extracted from cells and subjected to RNase treatment before standard bisulfitie conversion (Bisulfitie Kit, Promega). Bisulfitie DNA was subjected to PCR, and then a second nested PCR, to amplify the 3′-UTR of ER\(\alpha\)-1 using the method and primers previously described to amplify a DNA fragment of 644 bp. The PCR product was enzymatically digested using the Rsal restriction enzyme (New England Biolabs). Owing to a CG site in the native DNA within this amplicon, this digestion cuts methylated DNA amplicons into ~317 bp and ~327 bp fragments, leaving the non-methylated DNA uncut.

**shRNA experiments.** Two shRNA constructs to \(\text{Rb1}\) (TRC identifiers TRCN0000295892 and TRCN0000295842) and \(\text{Rb1}\) luciferase control were expressed in tumour cells as previously described. Suppression efficiency was assessed by RT-qPCR.

**Doxorubicin-induced senescence.** MDA-MB-453 and BT474 cells were treated with doxorubicin (Sigma Aldrich, 200 nM) for a period of 24 h. The cells were then cultured in fresh media for 72 h after treatment, and RNA was extracted for qPCR.
In vitro regulatory T-cell differentiation. CD4+CD25− T cells were isolated from spleens and lymph nodes of naive FVB mice using a CD4+CD25− Regulatory T Cell Kit (Miltenyi Biotec) with an autoMACs Pro Separator. Cells were cultured for 72 h in T-cell medium with CD3/CD28 Dynabeads (1:1 ratio of cells/beads, ThermoFisher), 100 U ml−1 rhIL-2 (Peprotech), with or without 25 ng ml−1 rhTGF-β (R&D Systems) with DMSO or abemaciclib (125–1,000 nM). Differentiation status for each condition was determined by intracellular flow cytometry for FoxP3. Fold change in percentage differentiation with the addition of rhTGF-β was calculated for each condition and normalized to the DMSO control.

T-cell proliferation in vitro. CD4+CD25− and CD4+CD25+ T cells were isolated from spleens and lymph nodes of naive FVB mice using the CD4+CD25+ Regulatory T Cell Kit (Miltenyi Biotec); CD8+ T cells were isolated as described above. Isolated T cells were labelled with 5 μM CFSE (Biolegend) as described above. 1 × 10^5 cells were cultured in T-cell medium with CD3/CD28 beads (1:1 ratio of cells to beads), 100 U ml−1 rhIL-2 and DMSO or abemaciclib (250 or 500 nM) for 72 h at 37°C. CFSE-dilution was analysed at endpoint by flow cytometry.

Gene expression in isolated T-cell populations. CD8+, CD4+CD25−, and CD4+CD25+ T cells were isolated as described above from lymph nodes and spleens of naive FVB mice treated with vehicle or 90 mg per kg abemaciclib for 12 days. Purity of isolated populations was confirmed by flow cytometry. mRNA was obtained using a NucleoSpin RNA XS Kit (Macherey-Nagel) according to the manufacturer’s instructions. RT-qPCR was performed as described above.

Patient data. Gene expression data (GEO GSE93204) were obtained from biopsy samples from the NeoPalAna clinical trial (NCT01723774). Expression data were used in the log2-normalized form. We used GSEA software (Broad Institute, Massachusetts, USA) with the following settings for analysis: number of permutations: 1,000; collapse data set of gene symbols: true; permutation type: gene set; enrichment statistic: weighted; metric for ranking genes: Signal2Noise; gene list sorting mode: real; gene list ordering mode: descending; max size/exclude larger sets: 500; min size/exclude smaller sets: 15. Analysis was performed using the Hallmarks v6.0 GSEA signatures.

Statistical analyses. Statistical analyses were performed as described in the figure legend for each experiment. All statistical tests were two-sided, and non-parametric tests were used when variance was dissimilar between groups. All data are presented as mean ± s.d. unless otherwise noted in the legends. Differences were considered statistically significant at P ≤ 0.05. All data shown are representative two or more independent experiments, unless indicated otherwise.

Data availability. Transcriptomic data that support the findings of this study have been deposited in the Gene Expression Omnibus under primary accession code GSE99063. Source data for western blots and tumour measurements are provided in Supplementary Fig. 1 and Supplementary Table 2. All other data are available from the corresponding authors upon reasonable request.

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Extended Data Figure 1 | Tumour cell proliferation and expression of cell-cycle-related genes after CDK4/6 inhibition.

a, Immunohistochemistry for Ki-67 in MMTV-rtTA/tetO-HER2 tumours treated for 12 days with abemaciclib or vehicle; representative images (scale bar, 100 μm) and quantification (n = 7 tumours per group).

b, Expression of E2F transcription factors, S phase genes, and G2/M genes in MMTV-rtTA/tetO-HER2 tumours treated with abemaciclib for 12 days compared with vehicle (vehicle, n = 11; abemaciclib, n = 12 tumours).

c, d, GO terms with P < 0.05 (c) or GSEA terms significantly downregulated (d) by abemaciclib compared with vehicle in MMTV-rtTA/tetO-HER2 tumours (vehicle, n = 11; abemaciclib, n = 12 tumours). Unpaired two-tailed t-tests (a, b). Error bars, s.d. *P < 0.05, **P < 0.01.
Extended Data Figure 2 | CDK4/6 inhibition enhances antigen presentation. a, Antigen processing and presentation gene expression in MDA-MB-361 cells treated with 500 nM abemaciclib or DMSO (7 days; n = 3). b, Gene expression in cell lines treated with DMSO or palbociclib (7 days; n = 3). c, β2M/MHC-I flow cytometry in cell lines; grey, FMO control. (For MDA-MB-453, vehicle and abemaciclib, n = 2; palbociclib, n = 3. For MDA-MB-361, n = 3.) d, Gene expression in TCGA samples (CCND1 shallow deletion, n = 101; CCND1 diploid, n = 503; CCND1 gain, n = 203; CCND1 amplified, n = 153). e, H-2Kb SIINFEKL flow cytometry after 7 days of abemaciclib or DMSO (B16-OVA, n = 9; MMTV-PyMT-S2WTP3-OVA, n = 3). f, CD8⁺ T-cell proliferation in response to abemaciclib-pretreated tumour cells (n = 3). g, IFN-γ and TNF-α production in tumour cell/OT-1 assay by ELISA (n = 3). One-way ANOVA adjusted for multiple comparisons (c, d, f), unpaired two-tailed t-tests (a, b, e, g). Error bars, s.d. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Extended Data Figure 3 | Effects of CDK4/6 inhibition on breast cancer cell proliferation and apoptosis in vitro. a, Relative numbers of breast cancer cells cultured in 250 nM (MDA-MB-453) or 500 nM (MDA-MB-361, BT474) abemaciclib or DMSO for 11 days, followed by drug withdrawal (arrow). b, Representative senescence-associated β-galactosidase staining of MDA-MB-453 cells (left) and BT474 cells (right) treated with DMSO or abemaciclib (MDA-MB-453, 250 nM; BT474, 500 nM) for 0, 4, and 7 days. c, Western blot of SKBR3, BT474, MDA-MB-453, and MDA-MB-361 cells treated with DMSO, lapatinib, or abemaciclib for 48 h. d, Western blot of MDA-MB-453 cells pretreated with DMSO or abemaciclib (500 nM) for 0, 1, or 7 days before exposure to staurosorine (500 nM) for 4 h. For western blot source images, see Supplementary Fig. 1.
Extended Data Figure 4 | See next page for caption.
Extended Data Figure 4 | CDK4/6 inhibition increases IFN signalling.

a, b, Top-ranked GO terms (a) and expression of IFN-responsive transcription factors (b) in MDA-MB-361 cells treated with 500 nM abemaciclib or DMSO (7 days; n = 3). c, Expression of ISGs in MMTV-PyMT-S2WTP3 cells treated with DMSO or abemaciclib (500 nM, 7 days) (n = 3). d, Expression of ISGs in MDA-MB-361 and MCF7 cells treated with abemaciclib (100 nM), fulvestrant (100 nM), or the combination for 7 days (n = 3). e, Expression of ISGs in MDA-MB-453, MCF7, and MDA-MB-231 cells treated with abemaciclib or DMSO (7 days; n = 3). f, Expression of ISGs in PDX 14-07 tumours treated with abemaciclib or vehicle (21–28 days; vehicle, n = 4; abemaciclib, n = 2 tumours per group). g, Phospho- and total STAT1 in cells treated with 500 nM abemaciclib as indicated. h, Confirmation of p16-Flag overexpression in MDA-MB-453 and BT474 cells (left) and gene expression in these cell lines by qPCR (right) (n = 6). i, j, Gene expression changes in MMTV-rtTA/tetO-HER2 tumours from mice treated with vehicle or abemaciclib for 12 days (vehicle, n = 11; abemaciclib, n = 12 tumours per group). Relative expression of IFN-responsive T-cell chemotactants (i); relative expression of ISGs (j). k, Correlation of expression of Stat1 and Nlrc5 with genes involved in antigen processing and presentation in MMTV-rtTA/tetO-HER2 tumours. Blue dots, vehicle-treated tumours; red dots, abemaciclib-treated tumours. (Symbol r is the Pearson product-moment correlation coefficient.) Unpaired two-tailed t-tests (b, d–f, h–j) adjusted for multiple comparisons (c). Error bars, s.d. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. For western blot source images, see Supplementary Fig. 1.
Extended Data Figure 5 | CDK4/6 inhibitors enhance expression of immune-related signatures in breast cancer patients.

a–c. NeoPalAna schema (a). 'Endo. Rx', endocrine therapy for breast cancer. Downregulated GSEA signatures after 2 weeks of palbociclib treatment (b); nominal $P < 0.001$, false discovery rate $q < 0.001$.

Upregulated GSEA signatures after 2 weeks palbociclib treatment (c); nominal $P < 0.001$, false discovery rate $q < 0.001$ (C1D1, $n = 34$; C1D15, $n = 29$; surgery, $n = 23$).
Extended Data Figure 6 | CDK4/6 inhibition mediates type III IFN production. a, Phospho- and total STAT1 in MDA-MB-453 cells treated with abemaciclib with or without ruxolitinib for 7 days. b, Effect of neutralization of IFN-α or IFN-γ on STAT1 mRNA expression (n = 2–4). c, d, Impact of neutralization of IFN-α (c) and IFN-γ (d) on phospho- and total STAT1 protein in indicated cell lines. e, Expression of type III IFN genes in indicated cell lines treated with abemaciclib for 7 days compared with DMSO (n = 3). f, Type III IFN production measured by ELISA (7 days; n = 2). Unpaired two-tailed t-tests (e, f) adjusted for multiple comparisons (b). Error bars, s.d. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. For western blot source images, see Supplementary Fig. 1.
Extended Data Figure 7 | DNMT1 suppression mediates dsRNA response. a, DNMT1 expression after treatment with abemaciclib (n = 3). b, DNMT1 protein expression after treatment with abemaciclib. c, DNMT3A expression after treatment with abemaciclib (500 nM) for 7 days (n = 3, except abemaciclib 24 h, n = 2). d, DNMT1 expression after abemaciclib or control in MMTV-rtTA/tetO-HER2 tumours, P = 0.05 (12 days; vehicle, n = 11; abemaciclib, n = 12 tumours per group). e, RB1 knockdown in MDA-MB-453 cells and its effect on indicated gene expression after 7 days of abemaciclib (500 nM) (two biological replicates each associated with three technical replicates). f, ERV3-1 methylation. g, ERV expression after abemaciclib or DMSO (7 days; n = 3). h, Relative dsRNA expression after 7 days of abemaciclib compared with DMSO (n = 3). i, j, Cytosolic pattern recognition receptors in cells (i) (7 days; n = 3) or PDX tumours (j) (21–28 days; vehicle, n = 4; abemaciclib, n = 2 tumours per group). k, Western blot of overexpression of DNMT1 in MDA-MB-453 cells and quantification of mRNA expression (n = 3). Unpaired two-tailed t-tests (d, e, g–k) adjusted for multiple comparisons (a). Error bars, s.d. *P ≤ 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. For western blot source images, see Supplementary Fig. 1.
Extended Data Figure 8 | Abemaciclib induces a ‘senescence-like’ phenotype without evidence of SASP. **a**, Representative senescence-associated β-galactosidase staining (left) of MMTV-rtTA/tetO-HER2 tumours treated with vehicle or abemaciclib for 12 days (scale bar, 500 μm). Quantification of relative senescence-associated β-galactosidase positive area (right, n = 6 tumours per group). **b**, mRNA expression of SASP factors in MMTV-rtTA/tetO-HER2 tumours treated as in Fig. 1a. Il6 expression determined by qPCR (n = 10 tumours per group); Il1a and Il1b expression determined by transcriptome analysis (vehicle, n = 11; abemaciclib, n = 12 tumours per group). **c**, MDA-MB-453 and BT474 cells treated with DMSO or abemaciclib (500 nM) for 7 days, and expression determined by qPCR. **d**, mRNA expression of Il6 upon doxorubicin-induced senescence (n = 3). MDA-MB-453 and BT474 cells were treated with doxorubicin (200 nM) for 24 h, and mRNA extracted 3 days later for qPCR. Unpaired two-tailed t-tests (a, d). Error bars, s.d. **P < 0.01. © 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.
Extended Data Figure 9 | See next page for caption.
Extended Data Figure 9 | Impact of CDK4/6 inhibition on immune cell populations and T<sub>Reg</sub> biology. a, Flow cytometric analysis of immune cell populations in MMTV-rtTA/tetO-HER2 tumours treated with vehicle or abemaciclib for 12 days (vehicle, n = 15; abemaciclib, n = 17 tumours per group). b, Peripheral blood T<sub>Reg</sub> cells in MMTV-rtTA/tetO-HER2 mice (n = 4 mice per group). c, d, T<sub>Reg</sub> cells (CD4<sup>+</sup>FoxP3<sup>+</sup>) quantified by flow cytometry of MMTV-PyMT tumours (c, vehicle, n = 18; abemaciclib, n = 16 tumours per group) and CT-26 tumours (d, n = 12 tumours per group) treated as indicated for 12 days. e, T<sub>Reg</sub> cells in lymph nodes of tumour-free mice (12 days; one-way ANOVA corrected for multiple comparisons, vehicles and palbociclib, n = 8; abemaciclib, n = 7 mice per group). f, T<sub>Reg</sub>-CD8<sup>+</sup> ratio in the spleens and lymph nodes (LN) of tumour-free FVB mice (12 days; vehicles and palbociclib, n = 8; abemaciclib, n = 7 mice per group). g, Plasma autoantibodies in tumour-free and tumour-bearing mice treated with vehicle or abemaciclib for 12 days (tumour-free vehicle, n = 8; tumour-free abemaciclib, n = 7; tumour-bearing vehicle, n = 7; tumour-bearing abemaciclib, n = 6 mice per group). h–l, Tumour-free FVB mice treated with abemaciclib or vehicle for 12 days. Thymic mass (h). Thymic cell populations were quantified by flow cytometry: CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) thymocytes (i); CD4<sup>+</sup> single-positive (SP) thymocytes (j); CD8<sup>+</sup> SP thymocytes (k); CD4<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells (l). (Vehicles and palbociclib, n = 5; abemaciclib, n = 4 mice per group.) m, Effect of abemaciclib or DMSO on ex vivo differentiation of CD4<sup>+</sup>CD25<sup>+</sup> T cells into T<sub>Reg</sub> cells in the presence of TGF-β for 72 h (n = 4). n, Effect of DMSO or abemaciclib treatment for 72 h on T<sub>Reg</sub> apoptosis measured by Annexin V staining (n = 2). o, Quantification of immunofluorescent staining of MMTV-rtTA/tetO-HER2 tumours (n = 7 tumours per group). p, q, Dnmt1 (p) and Cdkn1a (q) in T cells from tumour-free mice (12 days; two-way ANOVA corrected for multiple comparisons, n = 7 mice per group (pooled)). Error bars, s.d. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Extended Data Figure 10 | See next page for caption.
Extended Data Figure 10 | Role of adaptive immunity in response to abemaciclib and combination with immunotherapy. a, Relative change in volume of MMTV-rtTA/tetO-HER2 tumours implanted into Foxn1nu mice and then treated with vehicle (n = 11 tumours) or abemaciclib (n = 9 tumours) (one-way ANOVA). b, Immunohistochemistry of tumours in a for Ki-67. Representative images (left), and quantification of percentage of Ki-67+ cells (right) (vehicle, n = 7; abemaciclib, n = 10 per group; scale bar, 100μm). c, Flow cytometric analysis of absolute number (left) and percentage (right) of CD8+ T cells in peripheral blood after administration of anti-CD8 or isotype control antibodies (isotype, n = 6; anti-CD8, n = 4 mice per group). d–g, Expression of inhibitory co-receptors on intratumoural CD8+ T cells in MMTV-rtTA/tetO-HER2 tumours after 6 days of treatment with abemaciclib (n = 6 tumours) or vehicle (n = 5 tumours). PD-1 cell-surface expression (d); representative flow cytometry plots (left), quantification (right). Representative flow cytometry plots for CTLA-4 (e) and LAG3 (f). g, Quantification of e and f. h, Quantification of number of inhibitory receptors per cell on intratumoural CD8+ T cells in MMTV-PyMT tumours after treating mice with abemaciclib or vehicle (12 days; n = 18 tumours per group). i, Ifng in MMTV-rtTA/tetO-HER2 tumours (12 days; Mann–Whitney test, n = 10 tumours per group). j, Experimental schema for Fig. 4j. k, Quantification of mean change in tumour volume at d19 (top) and maximal reduction in tumour volume (bottom) of curves in Fig. 4j. l, Spider plots of CT-26 tumour growth with indicated treatments (n = 4 per group; one experiment). m, Kaplan–Meier curves of incidence of CT-26 tumour formation after re-challenge (n = 4 per group). n, Schematic. Unpaired two-tailed t-tests (b, c), two-way ANOVA corrected for multiple comparisons (d, g). Error bars, s.d.; except (a), s.e.m. *P < 0.05, **P < 0.001, ****P < 0.0001. For source data, see Supplementary Table 2.
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Experimental design

1. Sample size
   Describe how sample size was determined.
   For in vivo experiments, samples sizes were determined based on previous experience with the models utilized, including experience in variability of tumor growth.

2. Data exclusions
   Describe any data exclusions.
   No data were excluded from analysis.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   Experimental findings were reliably reproduced.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   Prior to treatment initiation, mice were randomized into groups of equal average tumor volume.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   In vivo experiments were performed in a blinded fashion when possible. Downstream analyses of mouse tissue (immunohistochemistry, image analysis, flow cytometry) all experiments were performed in a blinded fashion such that the people conducting and/or analyzing the assay were not aware of treatment groups until data gathering was complete.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   n/a

   Confirmed

   □ □ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   □ □ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   □ □ A statement indicating how many times each experiment was replicated
   □ □ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   □ □ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   □ □ The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
   □ □ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   □ □ Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
7. Software

Describe the software used to analyze the data in this study.

No custom code was used.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

There are no restrictions on availability of materials.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Immunostaining antibodies: Ki-67 (Vector), HER2 (AB16901; Abcam), STAT1 (AB47425; Abcam), FoxP3 (clone FJK-16s; eBioscience 14-5773-80), CD8 (clone 4SM15; eBioscience 14-0808-82), and Ki-67 (clone SP6; Thermo Scientific MA5-14520). Immunostaining secondary antibodies: AF488 AffiniPure donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories 715-545-150), Cy3 AffiniPure donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories 711-165-152), AF488 donkey anti-rabbit IgG (Life Technologies R37118), and AF647 goat anti-rat IgG (Life Technologies A21247). Western blotting antibodies: cleaved PARP (CST 9541), cleaved caspase-3 (CST 9661), phospho-STAT1 Y701 (CST 9167), STAT1 (CST 9172), FLAG (CST 2368), vinculin (V9131, Sigma) and DNMT1 (92314, Abcam). Murine flow cytometry antibodies: Antibodies were purchased from Biolegend unless otherwise indicated: CD45 (clone 30-F11; 103126 and 103131), CD3 (clone 145-2C11; 100320), CD8 (clone S3-6.7; 100714, 100743), CD4 (clone RM4-5, 100529), PD-1 (clone 29F.1A12; 135223), Tim-3 (clone RMT3-23; 119705), CTLA-4 (clone UC10-4B9; 106313), LAG-3 (clone C9B7W; 125207), B220 (clone RA3-682; 103245), NK1.1 (clone PK136; 108739), CD11b (clone M1/70; 101206), Ly6G (clone 1A8; 127639), Ly6C (clone AL-21; BD Pharmigen 560595), and FoxP3 (clone FJK-16s; eBioscience 17-5773-82). Human flow cytometry antibodies: 2-microglobulin (clone 2M2; 316304) and HLA-A,B,C (clone W6/32; 311425). dsRNA was detected by the K1 antibody (English and Scientific Consulting (Hungary). All antibodies were previously validated by their manufacturers.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

BT474, SKBR3, MDA-MB-361, MDA-MB-453, and MCF7 human breast cancer cell lines were obtained from ATCC. MMTV-PyMT-S2WTP3 cells were a gift from Dr. Andreas Möller, QIMR Berghofer Medical Research Institute, and CT-26 cells were a gift from Dr. Steve Elledge, Harvard University.

b. Describe the method of cell line authentication used.

All cell lines were authenticated by short tandem repeat analysis.

c. Report whether the cell lines were tested for mycoplasma contamination.

All cell lines tested negative for mycoplasma.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines were used.
Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

MMTV-rtTA/tetO-HER2 mice were maintained in house. Female FVB MMTV-PyMT, Balb/c (6-7 weeks old), and Foxn1nu (8 weeks old) mice were purchased from Jackson Labs. Female FVB mice (7 weeks old) were purchased from Taconic Biosciences. P14-TCR transgenic mice (gift from Dr. Kai Wucherpfennig, Harvard Medical School), and OT-I mice (C57BL/6 Tg(TcraTcrb)1100Mjb/J; Jackson Labs) were used as a source of CD8+ T cells for in vitro co-culture assays. FVB CD45.2+ mice (gift from Dr. Daniel Tenen, Harvard Medical School) were used as a source of T cells for in vitro studies.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

This study did not involve human research participants.
Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

Data presentation

For all flow cytometry data, confirm that:

☑ 1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☑ 2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).

☑ 3. All plots are contour plots with outliers or pseudocolor plots.

☑ 4. A numerical value for number of cells or percentage (with statistics) is provided.

Methodological details

5. Describe the sample preparation. Tumor cell lines –1x10⁶ cells per condition were stained with the appropriate antibodies diluted in PBS (Hyclone) plus 2% FBS (Life Technologies) for 30 minutes on ice. Matched fluorescence minus one (FMO) staining for each condition was performed as a control. Blood – Obtained by retro-orbital sampling at intermediate time points and by cardiac puncture at experimental endpoints. Blood cells and plasma were separated by centrifugation at 1,500 x g for 8 minutes at 4°C. Spleen, thymus, and lymph nodes – Single cell suspensions were obtained by mechanical digestion. Tumor - Tumors were first mechanically disrupted by chopping, then chemically digested in dissociation buffer (2 mg/mL collagenase type IV (Worthington Biochemical), 0.02 mg/mL DNase (Sigma Aldrich) in DMEM (Life Technologies) containing 5% FBS (Life Technologies), PenStrep (Hyclone)) with agitation at 37°C for 45 minutes. Following RBC lysis if necessary (blood, spleen, thymus, tumor; PharmLyse, BD Biosciences), single cell suspensions were blocked with anti-CD16/32 (Biolegend) for 20 minutes on ice and then incubated with appropriate antibodies for 30 min on ice.

6. Identify the instrument used for data collection. Flow cytometry was performed on a LSR II SORP 5 Laser Analyzer (BD Biosciences) or BD FACSCanto II Flow Cytometer (BD Biosciences, Ref. 338960).

7. Describe the software used to collect and analyze the flow cytometry data. FACSDiva software (BD Biosciences) was used to collect the data, and the data was analyzed using FlowJo (TreeStar).

8. Describe the abundance of the relevant cell populations within post-sort fractions. No FACS sorting was performed for this work.

9. Describe the gating strategy used. For all experiments, debris was first excluded by a morphology gate based on FSC-A and SSC-A. Then, non-singlets were eliminated from analysis by a single cell gate based on FSC-H and FSC-A. Next, dead cells were eliminated by an appropriate viability gate: 7AAD was used to distinguish live/dead cells, except in cases requiring intracellular staining in which case eFluor 450 (eBioscience) or Zombie Yellow (Biolegend) fixable viability dyes were used. When appropriate, all lymphocytes were identified using a CD45+ gate. For identification of regulatory T cells, CD45+ cells were gated for CD3+ CD4+ FoxP3+ cells. For analysis of checkpoint inhibitor
expression, CD3+ CD4+ and CD3+ CD8+ populations were then examined for PD-1, CTLA-4, Tim-3, and LAG-3 expression.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ☒