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Acquired IFN γ resistance impairs anti-tumor immunity and gives rise to T-cell-resistant melanoma lesions

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Melanoma treatment has been revolutionized by antibody-based immunotherapies. IFN γ secretion by CD8⁺ T cells is critical for therapy efficacy having anti-proliferative and pro-apoptotic effects on tumour cells. Our study demonstrates a genetic evolution of IFN γ resistance in different melanoma patient models. Chromosomal alterations and subsequent inactivating mutations in genes of the IFN γ signalling cascade, most often *JAK1* or *JAK2*, protect melanoma cells from anti-tumour IFN γ activity. *JAK1/2* mutants further evolve into T-cell-resistant HLA class I-negative lesions with genes involved in antigen presentation silenced and no longer inducible by IFN γ . Allelic *JAK1/2* losses predisposing to IFN γ resistance development are frequent in melanoma. Subclones harbouring inactivating mutations emerge under various immunotherapies but are also detectable in pre-treatment biopsies. Our data demonstrate that *JAK1/2* deficiency protects melanoma from anti-tumour IFN γ activity and results in T-cell-resistant HLA class I-negative lesions. Screening for mechanisms of IFN γ resistance should be considered in therapeutic decision-making.

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Understanding the mechanisms of T-cell inhibition by melanoma cells allowed for the development of new agents with considerable activity against metastatic disease including antibodies targeting the PD-L1/PD1 axis. PD-L1 expressed on melanoma cells binds its inhibitory PD1 receptor on cytotoxic CD8⁺ T lymphocytes generating a checkpoint signal dampening the T cell's effector function¹. Release from checkpoint blockade by treatment with anti-PD1 antibodies yields clinical benefit in a substantial proportion of melanoma patients, experiencing durable disease stabilization, tumour regression as well as complete remission^{2–4}. Response to anti-PD1 therapy is strongly associated with the expression of its ligand on melanoma cells and the presence of CD8⁺ T cells in the margin or center of metastatic lesions⁵. How T cells mediate disease stabilization or regression of bulky tumour masses remained unclear so far.

Upon activation by cognate HLA class I antigen complexes, T cells release cytolytic granules, containing perforins and granzymes, onto their target cells and secrete interferon (IFN) γ acting on cells in the microenvironment⁶. Perforin/granzyme-mediated killing and induction of apoptosis by death receptor engagement have long been considered the major anti-tumour effector mechanisms of CD8⁺ T cells. Accordingly, expression of cytolytic markers in pretreatment melanoma biopsies was found to be significantly associated with clinical benefit to antibodies targeting the T-cell checkpoint CTLA-4 (ref. 7). But evidence from different *in vivo* studies suggests that the anti-proliferative and pro-apoptotic activity of IFN γ on melanoma cells contributes essentially to the efficacy of T-cell-mediated anti-tumour immunity.

IFN γ binds to the heterodimeric IFNGR1/IFNGR2 receptor complex, leading to the activation of the receptor-associated kinases JAK1 and JAK2 that in turn phosphorylate STAT1. Phosphorylated STAT1 homodimers activate transcription of primary response genes including the transcriptional activator IRF1 that in turn coordinates the expression of secondary response genes⁸. Activation of the JAK1/2-STAT1-IRF1 signalling cascade in melanoma cells as well as other tumour cells can induce growth arrest and death via different pathways^{9–12}. Recently, it was demonstrated that adoptively transferred tumour antigen-specific CD8⁺ T cells infiltrating B16 melanoma lesions at low numbers arrested the growth of several times higher numbers of tumour cells in an IFN γ -dependent manner¹¹. Furthermore, T-cell-derived IFN γ in combination with tumour-necrosis factor (TNF) α was found to be essential also for *in vivo* induction of tumour-cell senescence abrogating disease progression in a pancreatic tumour model^{13,14}.

Based on this knowledge, we postulated that melanoma cells from patients responding to immunotherapy should be sensitive to the anti-proliferative and pro-apoptotic effects of IFN γ and that continuous cytokine exposure should select for the outgrowth of IFN γ -resistant tumour subclones. Here we demonstrate that IFN γ -resistant melanoma clones with

inactivating JAK1/JAK2 mutations frequently evolve in patients receiving different types of immunotherapy. IFN γ -resistant tumour cells are protected from cytokine-induced growth inhibition and apoptosis. Additionally, JAK1/JAK2-deficient lesions become T-cell-resistant by silencing HLA class I antigen presentation, which can no longer be restored by IFN γ signalling. Our findings suggest sequential screening of tumour biopsies for genetic defects in the IFN γ signalling cascade will aid therapeutic decision-making in patients with advanced melanoma.

Results

Acquired mutations in genes of the IFN γ signalling pathway.

Assuming that the growth-inhibitory and pro-apoptotic activity of T-cell-derived IFN γ acts selectively on tumour cells, the evolution of genetic variants in melanoma with impaired cytokine signalling was explored. In a first step, we evaluated available exome data of 46 melanoma cell lines¹⁵, established from metastases of different patients in our laboratory, for aberrations in *IFNGR1*, *IFNGR2*, *JAK1*, *JAK2*, *STAT1* and *IRF1*. Mutations in *JAK1* ($n = 3$), *JAK2* ($n = 1$) and *STAT1* ($n = 1$) were detected in 5 out of the 46 cell lines (Table 1). By Sanger sequencing we confirmed the mutations on freshly isolated DNA from the respective cell lines Ma-Mel-36, Ma-Mel-53, Ma-Mel-54a, Ma-Mel-85 and Ma-Mel-102. Independent of existing exome data, Sanger sequencing revealed a *JAK1* mutation in a cell line from melanoma patient Ma-Mel-61 (Table 1). The specific mutations present in the cell lines were also detected *in situ* in corresponding tumour tissue, with the exception of metastasis Ma-Mel-54a. As shown in Table 1, targeted sequencing revealed a homozygous status for the mutant allele in three of the six cell lines (Ma-Mel-54a, Ma-Mel-61g, Ma-Mel-102). To determine whether these mutations functionally impaired IFN γ signalling, the cell lines were treated with recombinant IFN γ for 48 h followed by protein expression analyses of pathway components and downstream targets.

Despite a *STAT1* (c.947C>T) mutation frequency of approximately ~100%, Ma-Mel-102 cells still showed a slight induction of pSTAT1 and IRF1 in the presence of IFN γ (Supplementary Fig. 1a–c). However, the signals were weak and detectable only after long-term exposure of X-ray films, suggesting that the S316L exchange, located between the coiled-coil and DNA binding domains, strongly decreased STAT1 protein stability without necessarily leading to its complete inactivation. Parallel analyses on Ma-Mel-85 cells revealed a strong IFN γ pathway activation compared with Ma-Mel-102 cells, accompanied with an elevated surface expression of CD54, HLA class I and PD-L1 (Supplementary Fig. 1b–d). This is in line with a *JAK1* (c.1548C>A) mutation frequency of only 50% (Supplementary Fig. 1a), suggesting that wild-type *JAK1* was still active in Ma-Mel-85 cells. As expected, IFN γ signalling was detected also in Ma-Mel-53 cells showing a *JAK1* (c.2338G>A) mutation frequency of only 24% (Supplementary Fig. 1a–c).

Table 1 | Acquired mutations in genes of the IFN γ signalling pathway.

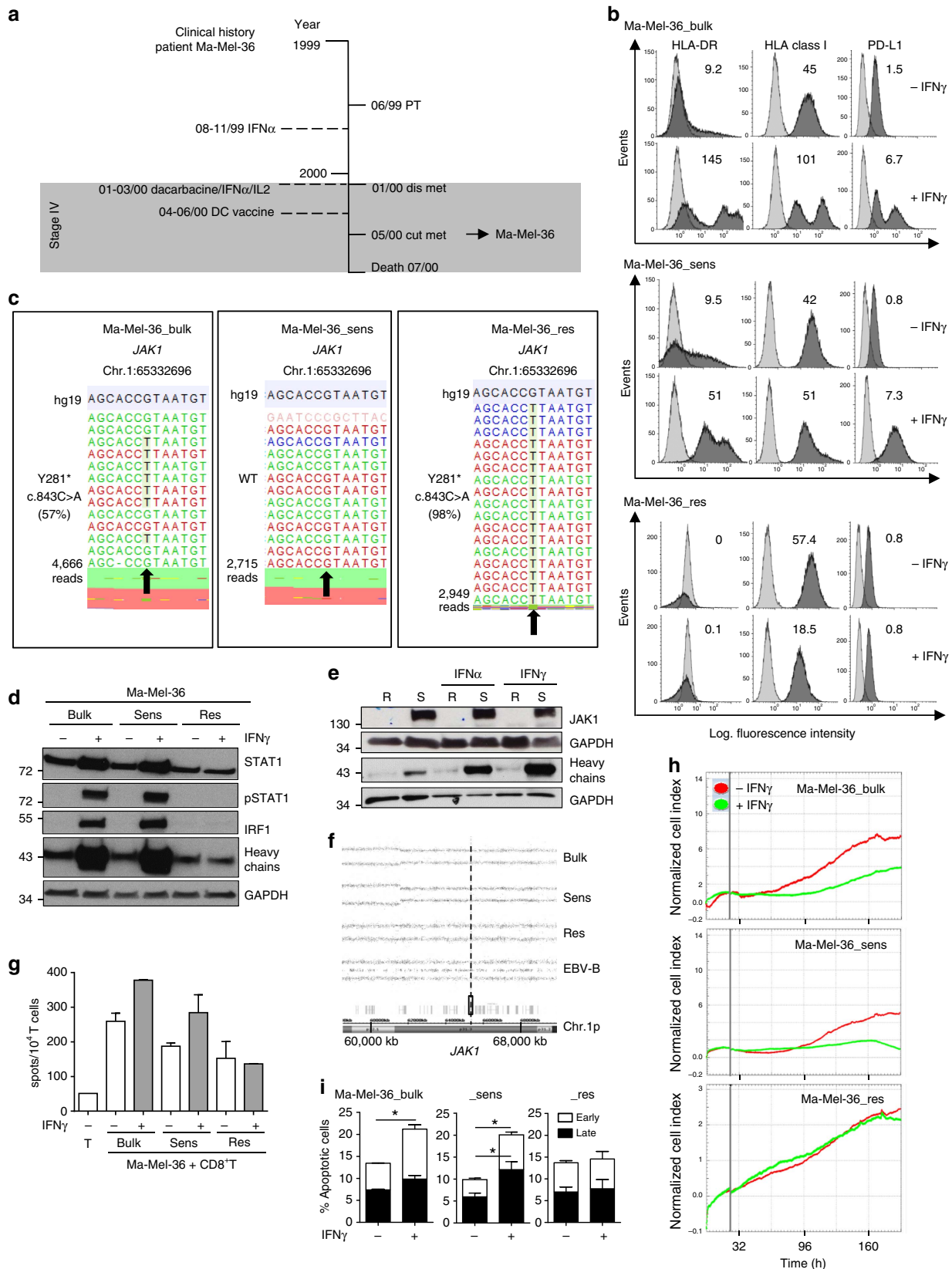
Patient	Metastasis excision	Disease stage	Cell line	Mutated gene	Allelic status	DNA	Protein	Mutation in tissue
Ma-Mel-36	05/2000	IV	Ma-Mel-36	<i>JAK1</i>	het*	c.843C>A	p.Y281*	Yes
Ma-Mel-53	03/2002	IV	Ma-Mel-53	<i>JAK1</i>	het	c.2338G>A	p.G780R	Yes
Ma-Mel-85	03/2004	IV	Ma-Mel-85	<i>JAK1</i>	het	c.1548C>A	p.F516L	Yes
Ma-Mel-54	09/2002	IV	Ma-Mel-54a	<i>JAK2</i>	hom	c.2876A>C	p.Q959P	n.d.
Ma-Mel-102	11/2004	III	Ma-Mel-102	<i>STAT1</i>	hom	c.947C>T	p.S316L	Yes
Ma-Mel-61	05/2005	IV	Ma-Mel-61g	<i>JAK1</i>	hom	c.1798G>T	p.G600W	Yes

n.d., not detectable.

*Consists in equal parts of homozygous wild-type and homozygous mutant subpopulations.

IFN γ resistance protects from cytokine-induced cell death. Targeted sequencing pointed to a heterozygous *JAK1* mutation in cell line Ma-Mel-36 (Table 1) that was established from a cutaneous patient metastasis (Fig. 1a). However, when treated with IFN γ , analyses of HLA-DR, HLA class I and PD-L1 surface

expression on Ma-Mel-36 cells (hereafter referred to as Ma-Mel-36_bulk cells) demonstrated that the cell line consisted in equal parts of IFN γ -sensitive and IFN γ -resistant subpopulations (Fig. 1b). Both subpopulations were sorted from IFN γ -treated Ma-Mel-36_bulk cells based on their different



HLA-DR expression profiles. The IFN γ -sensitive subpopulation Ma-Mel-36_sens strongly upregulated HLA-DR and PD-L1 surface expression in response to cytokine treatment, whereas the IFN γ -resistant subpopulation Ma-Mel-36_res remained HLA-DR-negative and PD-L1-low under these conditions (Fig. 1b). By targeted sequencing we detected the *JAK1* mutation (c.843C>A) encoding a truncated non-functional *JAK1*-Y281* variant in ~60 and 100% of Ma-Mel-36_bulk and Ma-Mel-36_res cells, respectively, but not in Ma-Mel-36_sens cells (Fig. 1c). Accordingly, IFN γ treatment resulted in pSTAT1 and IRF1 detection in lysates from Ma-Mel-36_bulk and Ma-Mel-36_sens cells but not from Ma-Mel-36_res cells (Fig. 1d). In line with the sequencing results, *JAK1* protein expression was detected only in Ma-Mel-36_sens but not in Ma-Mel-36_res cells (Fig. 1e).

Assuming that *JAK1* deficiency in these cells was due to a gene mutation and concurrent allelic loss, we performed single-nucleotide polymorphism (SNP) array analyses on DNA obtained from the three tumour cell populations and autologous Epstein-Barr virus (EBV)-transformed B cells as a control. The same partial deletion on chromosome 1p, encompassing the region 1p36.3-1p13.1 (Chr.1:854,277-116,804,754) including the *JAK1* gene mapping at Chr.1p31.3 was detected in Ma-Mel-36_bulk, Ma-Mel-36_sens and Ma-Mel-36_res cells (Fig. 1f). This result demonstrated that the allelic *JAK1* loss occurred early in the course of disease and that a subsequent c.843C>A mutation in the remaining *JAK1* allele generated the *JAK1*-deficient Ma-Mel-36_res subpopulation. Consistently, *JAK1* reconstitution by transient transfection of Ma-Mel-36_res cells with a *JAK1* expression plasmid restored IFN γ signalling (Supplementary Fig. 2a–c).

As shown in Fig. 1a, metastasis Ma-Mel-36 developed after the patient had been treated with recombinant IFN α and a combination of dacarbazine/IFN α /interleukin (IL) 2, suggesting activated tumour-reactive T cells selectively enriched the IFN γ -resistant cell subpopulation. Indeed, peripheral blood CD8⁺ T cells from patient Ma-Mel-36 secreted IFN γ in the presence of autologous melanoma cells, as determined by ELISpot assay (Fig. 1g). Pretreatment of tumour cells with IFN γ slightly enhanced the activation of CD8⁺ T cells by Ma-Mel-36_bulk and Ma-Mel-36_sens cells, whereas the T-cell-stimulatory capacity of Ma-Mel-36_res cells was not affected. Furthermore, impedance-based measurement of real-time proliferation in the xCELLigence system revealed a negative impact of IFN γ on the expansion of Ma-Mel-36_bulk and Ma-Mel-36_sens cells, while Ma-Mel-36_res cells efficiently proliferated (Fig. 1h). This was measurable also in terms of cell numbers: a considerable

reduction in Ma-Mel-36_bulk and in particular Ma-Mel-36_sens cells was noted in the presence of IFN γ , due to an increase in apoptosis (Fig. 1i and Supplementary Fig. 2d,e). In contrast, cell numbers and spontaneous apoptosis of IFN γ -resistant Ma-Mel-36_res cells remained unaffected under these conditions (Fig. 1i and Supplementary Fig. 2d,e).

As shown in Fig. 1e, Ma-Mel-36_sens cells responded to IFN α treatment whereas *JAK1*-deficient Ma-Mel-36_res cells were also resistant to type I IFN. Considering patient Ma-Mel-36 received IFN α -based therapies before the development of resistant lesions, we hypothesized that type I IFN signalling by affecting cell survival might have contributed to the enrichment of *JAK1*-deficient cells. However, in contrast to IFN γ , IFN α treatment did not affect the survival of Ma-Mel-36_sens cells (Supplementary Fig. 2f).

JAK2 deficiency blocks HLA class I upregulation by IFN γ . By Sanger sequencing we found a *JAK2* c.2876A>C exchange to be present in Ma-Mel-54a cells that, however, could not be detected in the corresponding tumour tissue (Table 1). To demonstrate that, in fact, the specific genetic alteration was acquired in the course of disease we sequenced DNA from a second cutaneous lesion (Ma-Mel-54b) of the patient, obtained one month after excision of metastasis Ma-Mel-54a (Fig. 2a). Indeed, tumour tissue Ma-Mel-54b and the corresponding cell line harboured the *JAK2* mutation already present in Ma-Mel-54a cells (Fig. 2b). Both cell lines showed a *JAK2* (c.2876A>C) mutation frequency of 100%, resulting in a Q959P exchange in the functionally important *JAK2* JH1 kinase domain¹⁶. As shown in Fig. 2c, mutant *JAK2*-Q959P was no longer detectable by western blot. Accordingly, IFN γ signalling was completely abrogated in both cell lines, which no longer showed CD54, HLA class I and PD-L1 upregulation in response to cytokine treatment (Fig. 2d,e).

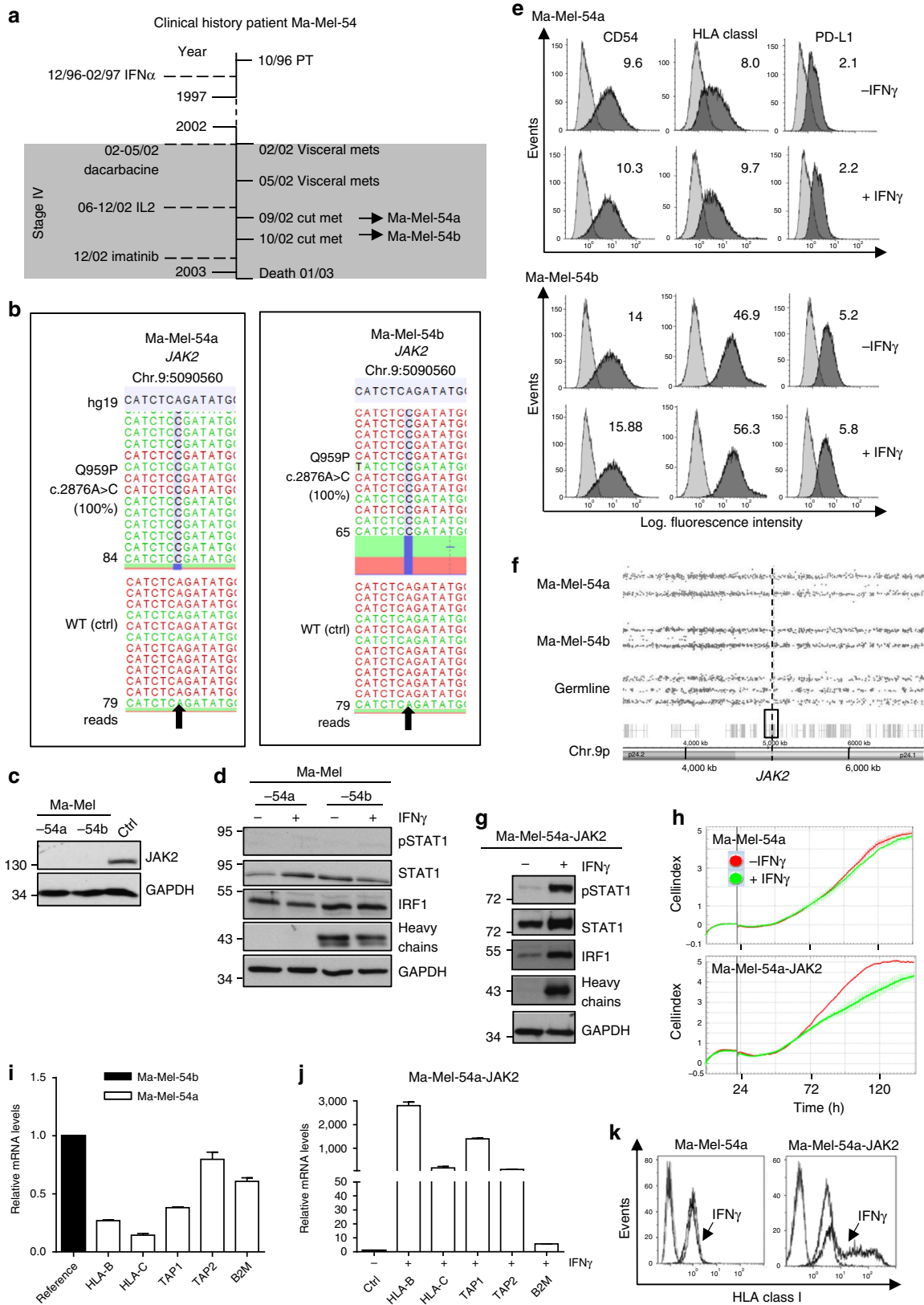
Assuming that *JAK2* deficiency of Ma-Mel-54 cells was caused by the co-occurrence of a *JAK2* gene mutation and allelic *JAK2* loss, we performed SNP array analyses on DNA obtained from the two cell lines and autologous peripheral blood cells as a constitutive, normal control to detect aberrations of chromosome 9p to which the *JAK2* gene maps at Chr.9p24.1. The same deletion on chromosome 9p, encompassing the region 9p24.3–p13.2 (Chr.9:203,861-37,578,327) was detected in Ma-Mel-54a and Ma-Mel-54b cells (Fig. 2f), demonstrating the common origin of *JAK2* deficiency in both metastases. As shown in Fig. 2g, IFN γ sensitivity of Ma-Mel-54a cells was restored upon transient *JAK2* re-expression as indicated by the induction of signalling pathway components. Furthermore, Ma-Mel-54a-*JAK2*

Figure 1 | Protection from cytokine-induced cell death by acquired IFN γ resistance. (a) Clinical history of patient Ma-Mel-36. Vertical line, time axis; left, therapeutic regimens; right, primary tumour (PT)/metastases development; arrow indicates cell line established from metastasis Ma-Mel-36; grey box, stage IV disease. (b) IFN γ -sensitive Ma-Mel-36_sens and IFN γ -resistant Ma-Mel-36_res cells sorted from IFN γ -treated (48 h) Ma-Mel-36_bulk cells based on their HLA-DR expression profile. Surface expression of indicated proteins measured by flow cytometry. Representative data from $n = 3$ independent experiments. (c) *JAK1* mutation defined by targeted sequencing on DNA from Ma-Mel-36_bulk and Ma-Mel-36_res cells. Plots of aligned sequencing reads in the location where the *JAK1* c.843C>A, p.Y281* mutation was identified, arrows highlight mutation or corresponding wild-type (WT) site. Number of sequencing reads notated on the left; %, frequency of mutation in reads. (d) Melanoma cells analysed by western blot for expression of STAT1, pSTAT1, IRF1 and HLA class I heavy chains after IFN γ treatment (48 h); GAPDH, loading control. Representative data from $n = 2$ independent experiments. (e) Ma-Mel-36_sens (S) and Ma-Mel-36_res (R) cells analysed for protein expression after IFN α and IFN γ treatment (48 h). Representative data from $n = 2$ independent experiments. (f) SNP results given as allelic distribution of chromosome 1p shown for DNA obtained from Ma-Mel-36_bulk, Ma-Mel-36_sens, Ma-Mel-36_res and autologous Epstein-Barr virus-transformed B cells as a control. Loss of one chromosomal allele in the region 1p36.3-1p13.1 (Chr.1:854,277-116,804,754; hg19) in all Ma-Mel-36 cell populations. Dashed line indicates *JAK1* location at Chr.1p31.3. (g) IFN γ release by autologous CD8⁺ T cells in the presence of IFN γ -treated (24 h) Ma-Mel-36 cell populations measured by ELISpot assay. Mean values (\pm s.e.m.) from $n = 2$ measurements. (h) Real-time proliferation of Ma-Mel-36 cell populations in the presence/absence of IFN γ . Bold grey vertical lines indicate addition of IFN γ . Representative data from $n = 3$ independent experiments. (i) IFN γ -induced (7 days) apoptosis in Ma-Mel-36 cell populations determined by AnnexinV/PI staining. Percentage of early (AnnexinV +/PI -) and late apoptotic (AnnexinV +/PI +) cells depicted. Mean values (\pm s.e.m.) from $n = 3$ independent experiments. Only statistical significant differences defined by paired Student's *t*-test are indicated, * $P < 0.05$.

transfectants proved to be sensitive towards the anti-proliferative activity of IFN γ in contrast to non-transfected Ma-Mel-54a cells (Fig. 2h).

Interestingly, western blot analyses suggested a lack of HLA class I heavy chain expression in Ma-Mel-54a compared with

Ma-Mel-54b cells (Fig. 2d). Indeed, Ma-Mel-54a cells only weakly expressed HLA class I surface molecules (Fig. 2e). By quantitative reverse transcription-PCR we demonstrated low level expression of specific messenger RNAs (mRNAs) involved in antigen presentation in Ma-Mel-54a cells (Fig. 2i). Expression of



HLA-B, *HLA-C*, *TAP1*, *TAP2* and *B2M* genes as well as HLA class I surface molecules were strongly upregulated solely by IFN γ -treated Ma-Mel-54a-JAK2 transfectants (Fig. 2j,k). Thus, JAK2 deficiency protected Ma-Mel-54a cells not only from anti-tumour IFN γ activity but also conserved their HLA class I-low phenotype that in turn might have hampered effective T-cell recognition of the tumour cells. Due to a lack of cryopreserved autologous T cells from this patient, the functional significance of the HLA class I-low phenotype could not be investigated in more detail.

Chromosome 1 alterations predispose to JAK1 deficiency.

Similar to Ma-Mel-54, multiple cell lines were established from distinct melanoma metastases of patient Ma-Mel-61 collected over a period of 2.5 years, allowing us to follow the development of IFN γ resistance in the course of disease. The patient presented in the clinic with stage IV melanoma at the end of 2002 and received IFN α treatment for more than 2 years from December 2002 to January 2005 (Fig. 3a). From three of his metastases, excised under IFN α treatment, the cell lines Ma-Mel-61a, Ma-Mel-61b and Ma-Mel-61c were established. Additional three lesions were resected in 2005 after IFN α therapy, giving rise to the cell lines Ma-Mel-61e, Ma-Mel-61g and Ma-Mel-61h (Fig. 3a). Treatment with IFN γ and subsequent analyses of CD54, HLA class I and PD-L1 surface expression by flow cytometry revealed an upregulation of these markers only for cell lines established before Ma-Mel-61g (Fig. 3b, and Supplementary Fig. 3a).

By targeted sequencing on DNA from Ma-Mel-61g and blood cells as a constitutive normal control we detected a *JAK1* c.1798G>T, *JAK1*-G600W mutation in 99% of the cells (Fig. 3c, Supplementary Fig. 3b), with the *JAK1*-G600W exchange affecting a conserved amino acid in the auto-inhibitory pseudokinase domain¹⁷. None of the IFN γ -sensitive Ma-Mel-61 cell lines showed a *JAK1* mutation (Fig. 3c, Supplementary Fig. 3b). The *JAK1*-G600W mutant protein was still detectable by western blot (Fig. 3d), but was found to be completely inactive, as no upregulation of pSTAT1, STAT1 and IRF1 was detected in Ma-Mel-61g cells in response to IFN γ treatment (Fig. 3e). Consistently, transfection of Ma-Mel-61g cells with an expression plasmid encoding wild-type *JAK1*, in contrast to *JAK1*-G600W, restored IFN γ signalling (Fig. 3f and Supplementary Fig. 3c). Again we detected autologous CD8⁺ T cells secreting IFN γ in the presence of Ma-Mel-61g cells in the patient's peripheral blood, whose activity might have selected for the outgrowth of IFN γ -resistant cells (Fig. 3g). Accordingly, while proliferation of Ma-Mel-61g cells was not affected by IFN γ , the cells became sensitive upon *JAK1* re-expression (Fig. 3h). Analysing the

genetic evolution of *JAK1* deficiency, we found a deletion on chromosome 1p, encompassing the region 1p34.3–1p12 (Chr.1:40,061,699–118,932,325) including the *JAK1* gene, to be present in all Ma-Mel-61 cell lines (Fig. 3i). This demonstrated that allelic *JAK1* loss was an early event in the course of disease progression in this patient, predisposing to IFN γ -resistance development, and that a subsequent inactivating *JAK1* point mutation led to complete abrogation of type II IFN signalling in Ma-Mel-61g cells.

Evolution of T-cell-resistant lesions. Similar to Ma-Mel-61g, IFN γ signalling was abrogated in Ma-Mel-61h cells due to the homozygous *JAK1* c.1798G>T, *JAK1*-G600W mutation (Fig. 4a,b). In contrast to Ma-Mel-61g, however, Ma-Mel-61h cells demonstrated a stable HLA class I-negative phenotype. Lack of HLA heavy chains and HLA class I surface expression was observed by western blot and flow cytometry, respectively (Fig. 4b,c). Staining of Ma-Mel-61g and Ma-Mel-61h tissue sections revealed the presence of HLA class I-negative tumour cells in both lesions (Fig. 4d and Supplementary Fig. 3d). Quantification of mRNAs involved in antigen presentation indicated a complete lack in the expression of *HLA-B*, *HLA-C*, *TAP1* and *TAP2* in Ma-Mel-61h cells compared with the control cells Ma-Mel-61b (Fig. 4e). Transient *JAK1* re-expression and subsequent IFN γ treatment induced *de novo* *HLA-B*, *HLA-C*, *TAP1* and *TAP2* mRNA expression demonstrating a reversible silencing of antigen presentation in Ma-Mel-61h cells (Fig. 4e). This could not be observed for Ma-Mel-61h cells expressing *JAK1*-G600W, confirming functional inactivity of the mutant protein. Consistently, enhanced expression of specific mRNAs could also be measured for IFN γ -treated Ma-Mel-61g-JAK1 but not for Ma-Mel-61g-JAK1-G600W transfectants (Fig. 4e). As shown in Fig. 4f, the subpopulation of Ma-Mel-61h-JAK1 transfectants demonstrated *de novo* HLA class I surface expression after IFN γ -treatment, resulting in detection of the previously ignored tumour cells by autologous CD8⁺ T cells (Fig. 4g). Overall, these data demonstrated that *JAK1* deficiency in Ma-Mel-61h cells was followed by silencing of antigen presentation, generating a T-cell-resistant melanoma phenotype. Similar results were obtained for *JAK2*-deficient Ma-Mel-54a cells (Fig. 2g,i,k), suggesting the broader significance of our findings.

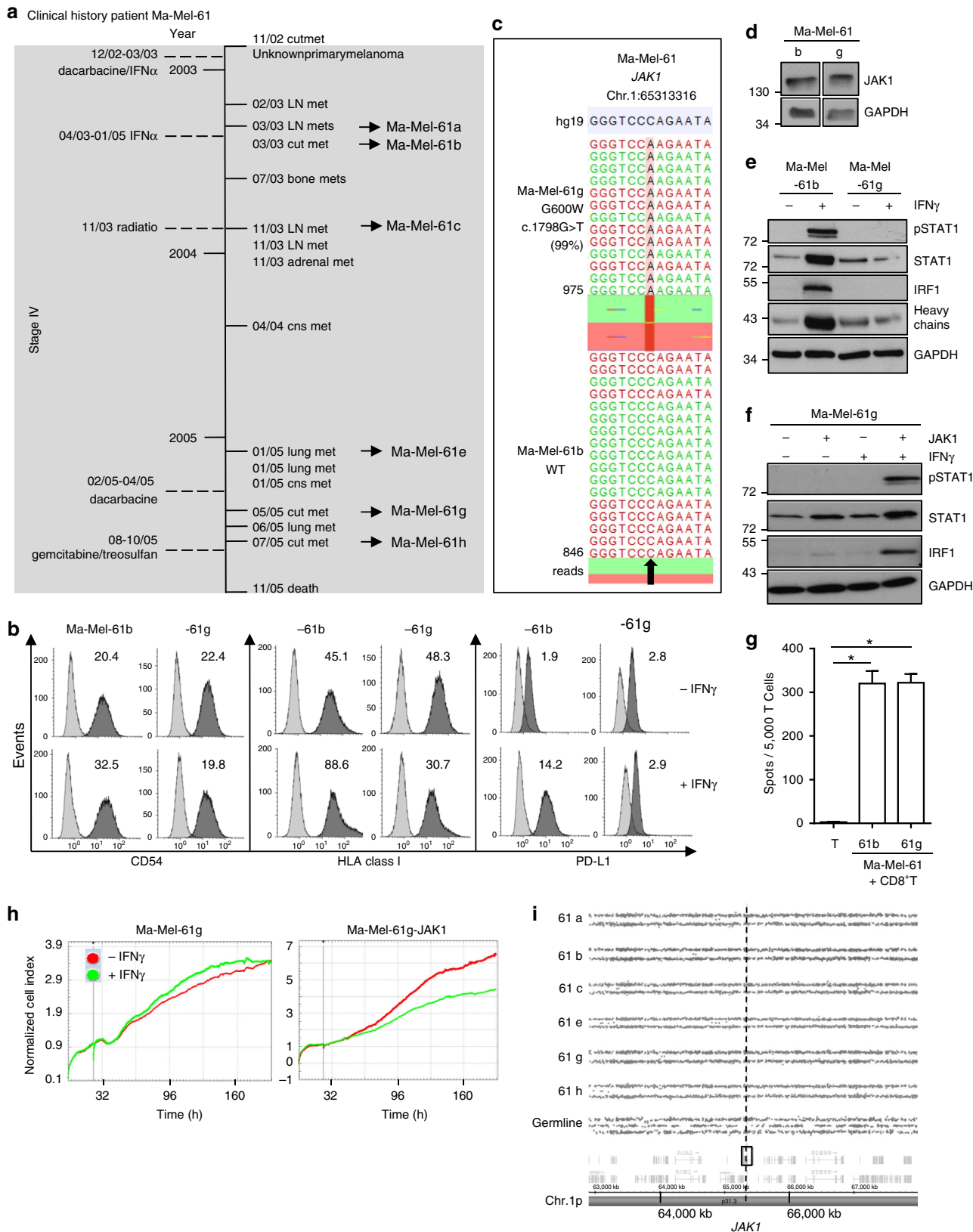
Genetic alterations defined in different melanoma data sets.

The detection of IFN γ -resistant melanoma metastases in our patient cohort led us to assess the presence of alterations in type II IFN signalling pathway components in independent sample

Figure 2 | JAK2 deficiency blocks HLA class I upregulation by IFN γ . (a) Clinical history of patient Ma-Mel-54. Vertical line, time axis; left, therapeutic regimens; right, primary tumour (PT)/metastases development; arrows indicate cell lines established from metastases Ma-Mel-54a and Ma-Mel-54b; grey box, stage IV disease. (b) Mutations defined by targeted sequencing on DNA from melanoma cells and autologous blood cells as wild-type (WT) control (ctrl). Plots of aligned sequencing reads in the location where the *JAK2* c.2876A>C, p.Q959P mutation was identified. WT sequences shown on the bottom, arrows highlight mutation sites. Number of sequencing reads notated on the left; %, frequency of mutations in reads. (c) Lysates from Ma-Mel-54a, Ma-Mel-54b and control cells (ctrl) analysed by western blot for *JAK2* expression. (d) Lysates from IFN γ -treated (48 h) Ma-Mel-54a and Ma-Mel-54b cells analysed for expression of STAT1, pSTAT1, IRF1 and HLA class I heavy chains. (c,d) GAPDH, loading control. Representative data from $n = 2$ independent experiments. (e) Expression of CD54, HLA class I and PD-L1 on IFN γ -treated (48 h) melanoma cells, measured by flow cytometry. Representative data from $n = 3$ independent experiments. (f) SNP results given as allelic distribution of chromosome 9p shown for DNA obtained from Ma-Mel-54a, Ma-Mel-54b and autologous peripheral blood cells as normal control (germline). Loss of one chromosomal allele in region 9p24.3–p13.2 (Chr.9:203,861–37,578,327; hg19) present in both cell lines. Dashed line indicates *JAK2* location at Chr.9p24.1. (g) Lysates from IFN γ -treated (48 h) Ma-Mel-54a-JAK2 transfectants analysed by western blot for expression of indicated proteins. (h) Real-time cell proliferation in the presence or absence of IFN γ . Bold grey vertical lines indicate addition of IFN γ . (g,h) Representative data from $n = 2$ independent experiments. (i) Ma-Mel-54a and Ma-Mel-54b cells analysed for specific mRNA expression by quantitative reverse transcription-PCR. (j) Ma-Mel-54a-JAK2 transfectants analysed for specific mRNA expression by quantitative reverse transcription-PCR in the presence or absence of IFN γ (48 h). (i,j) Relative expression levels given as means (\pm s.e.m.) from $n = 2$ independent experiments. (k) HLA class I expression on IFN γ -treated (48 h) Ma-Mel-54a cells and Ma-Mel-54a-JAK2 transfectants, measured by flow cytometry. Representative data from $n = 2$ independent experiments.

collections. We screened 287 TCGA (The Cancer Genome Atlas) melanoma tissue samples for mutations in *IFNGR1*, *IFNGR2*, *JAK1*, *JAK2*, *STAT1* and *IRF1* (ref. 18). In 12.6% (36 of 287) of the samples alterations were identified, including single-nucleotide variations (SNV), small insertions and deletions (Indel) and

homozygous deletions, that in most cases were mutually exclusive (Fig. 5a; Supplementary Data 1, Supplementary Fig. 4). Interestingly, a remarkable fraction of the biopsies (44%, 16 of 36) showed homozygous deletions in the different genes indicating abrogation of type II IFN signalling in tumour cells. Alterations



affecting genes of the IFN γ signalling cascade were more frequent in metastatic samples compared with primary tumours (Fig. 5b) and were detectable in metastases from patients receiving neo-adjuvant IFN α and from patients without treatment (Supplementary Data 1). Tumour biopsies with mutations did not show elevated expression of *IFNG* or *CD8A* mRNA in comparison to biopsies without mutations, suggesting a comparable activity of T cells in both types of lesions (Supplementary Fig. 5).

Support of our finding of recurrent genetic alterations in type II IFN signalling pathway genes was also obtained from additional published data sets. A mutation frequency of 22% (11 of 49), based on SNV/Indels and homozygous deletions, was detected in melanoma cell lines studied by the Cancer Cell Line Encyclopedia (CCLE)¹⁹. Considering only SNV/Indels, the frequency in melanoma tissue samples was 7% (20 of 287) for the TCGA melanoma collection (Fig. 5a)¹⁸, 3% (3 of 91) for melanoma biopsies studied by Krauthammer *et al.*²⁰ (Fig. 5c), 6% (3 of 49) for melanoma cell lines from CCLE¹⁹ (Fig. 5d) and 10% (12 of 121) for the cell lines analysed by Hodis *et al.*¹⁵ (Fig. 5e). Overall these analyses found mutations to be present in a considerable fraction of melanoma cells.

Our studies in the three melanoma patient models identified allelic *JAK1* and *JAK2* losses as initial genetic alteration predisposing to IFN γ -resistance development. This led us to screen available SNP microarray data from 59 'in-house' melanoma cell lines for loss of heterozygosity (LOH) in the *JAK1* and the *JAK2* locus²¹. As shown in Fig. 5f, LOH for *JAK1* was detected in 25% (15 of 59) and for *JAK2* in 76% (45 of 59) of the cell lines suggesting a high risk of resistance development in the course of an effective anti-tumour T-cell response.

Based on the above sequencing results and functional data, we asked for the impact of alterations in IFN γ signalling genes on the control of disease progression. When assessed in the largest available TCGA melanoma cohort with survival data (479 samples) these alterations were found to have a statistically significant negative impact on patient survival (Fig. 6a). On the other hand, elevated mRNA levels for *STAT1* as well as its downstream target *IRF1*, indicating active IFN γ signalling, were strongly associated with improved overall survival (Fig. 6b). The direct correlation between *IFNG* and *CD8A* mRNA expression pointed to CD8⁺ T cells as the major cytokine source (Supplementary Fig. 6a,b).

Mutations emerge before checkpoint blocking therapy. As resistance to IFN γ signalling could impact therapy efficacy, we screened 59 formalin-fixed, paraffin-embedded (FFPE) tumour samples from patients receiving anti-PD1 therapy for corresponding mutations by targeted sequencing of DNA isolated from

macrodissected tumour cells and autologous control blood cells (Table 2). In 19% of the biopsies (11 out of 59) mutations were identified, affecting *IRF1* ($n = 1$), *JAK1* ($n = 5$) and *JAK2* ($n = 5$), some of them clearly inactivating as the stop codon mutation in sample 15-12774 (*IRF1* W195*), and the frame shift mutations in specimens D5923-13 (*JAK2*-D710fs) and 18298-15 (*JAK2*-T376fs). Furthermore, we determined the mutation frequency in pre-treatment biopsies from patients having received anti-CTLA-4 therapy, in this case evaluating existing exome sequencing data⁷. Mutations in type II IFN signalling pathway genes were identified in 9% of samples (10 out of 110) (Table 2).

Overall, these sequencing results demonstrate that genetic alterations in type II IFN signalling pathway components are present in a considerable number of melanomas. Although we could not detect significant differences in responses to anti-PD1 or anti-CTLA-4 treatment between patients with or without mutations (Supplementary Table 1), it is important to note that two of the three patients with clearly inactivating mutations (15-12774: *IRF1* W195*; 18298-15: *JAK2* D710fs) showed progressive disease under anti-PD1 treatment, while the third patient (18298-15: *JAK2* T376fs) showed a partial response, suggesting that these mutations might have contributed to therapy resistance. Considerably larger cohorts of anti-PD1 and anti-CTLA-4 treated patients will be required to allow conclusive statistical analysis to be performed in future studies.

Discussion

Remarkable response rates in treatment of metastatic melanoma have been reported for adoptive T-cell transfer^{22,23} and therapy with immune checkpoint-blocking antibodies, including anti-PD1 monotherapy^{2,5} as well as anti-PD1 and anti-CTLA-4 combination therapy^{4,24}. Long-term data from anti-CTLA-4 therapy suggest that a number of patients will show a durable complete response and may even be healed of metastatic disease²⁵. Despite the considerable therapeutic potential, not all patients benefit equally well from immunotherapy. Primary as well as acquired therapy resistance is a major concern and the identification of resistance mechanisms is crucial for advancing treatment of melanoma and other malignancies.

The data presented in this work signify that under the selective pressure of an effective T-cell response tumour clones evolve that are considerably less susceptible or even resistant to T-cell effector mechanisms. As such the direct cytotoxic effects of CD8⁺ T cells mediated by release of cytolytic granules or death receptor engagement are an essential but most likely insufficient part of the overall anti-tumour response that also depends on the secretion of IFN γ . By induction of growth arrest and cell death IFN γ has a broader impact on tumour cells and their

Figure 3 | Chromosome 1 alterations predispose to JAK1 deficiency. (a) Clinical history of patient Ma-Mel-61. Vertical line, time axis; left, therapeutic regimens; right, metastases development; arrows indicate cell lines established from metastases Ma-Mel-61a, Ma-Mel-61b, Ma-Mel-61c, Ma-Mel-61e, Ma-Mel-61g and Ma-Mel-61h; grey box, stage IV disease. (b) Surface expression of CD54, HLA class I, and PD-L1 on IFN γ -treated (48 h) Ma-Mel-61b and Ma-Mel-61g cells, measured by flow cytometry. Representative data from $n = 3$ independent experiments. (c) Mutation defined by targeted sequencing on DNA from Ma-Mel-61g cells. Plots of aligned sequencing reads in the location where the *JAK1* c.1798G > T, p.G600W mutation was identified. Arrow highlights mutation site in Ma-Mel-61g or corresponding wild-type site (WT) in Ma-Mel-61b cells. Number of sequencing reads notated on the left; %, frequency of mutation in reads. (d) Lysates from Ma-Mel-61b and Ma-Mel-61g cells analysed by western blot for *JAK1* expression; GAPDH, loading control. Representative data from $n = 3$ independent experiments. (e) Lysates from IFN γ -treated (48 h) melanoma cells analysed by western blot for expression of *STAT1*, p*STAT1*, *IRF1* and HLA class I heavy chains; GAPDH, loading control. Representative data from $n = 3$ independent experiments. (f) Lysates from IFN γ -treated (48 h) *JAK1*-transfected Ma-Mel-61g cells analysed for expression of the indicated proteins. Representative data from $n = 3$ independent experiments. (g) IFN γ release by autologous CD8⁺ T cells in the presence of melanoma cells, measured by ELISpot assay. Means and s.e.m. (error bars) from $n = 4$ independent measurement. Statistical significant differences defined by paired Student's *t*-test are indicated, * $P < 0.05$. (h) Ma-Mel-61g and Ma-Mel-61g-*JAK1* cells subjected to impedance-based real-time measurement of proliferation in the presence or absence of IFN γ . Addition of IFN γ indicated by bold grey vertical lines. Representative data from $n = 3$ independent experiments. (i) SNP results given as allelic distribution of chromosome 1p shown for DNA obtained from the different Ma-Mel-61 cell lines and autologous blood cells as normal control (germline). Loss of one chromosomal allele in the region 1p34.3-1p12 (Chr.1:40,061,699-118,932,325; hg19) present in all cell lines. Dashed line indicates location of *JAK1* at Chr.1p31.3.

Assuming that IFN γ exerts a strong selective pressure on tumour cells we screened an ‘in-house’ collection of short-term cultured melanoma cell lines for mutations in genes of the IFN γ signalling pathway and detected *JAK1*, *JAK2* and *STAT1* alterations in cells and corresponding tumour tissue from 6 out of 47 patients. In two heterozygous *JAK1* mutants IFN γ signalling was still active but was strongly impaired and no longer detectable in homozygous *STAT1* and *JAK1/2* mutants, respectively. *JAK1/2*-deficient tumour cells emerged in disease stage IV metastases under/after immunotherapy. The different treatments, including IFN α , IL2 and combinations thereof, might have induced or boosted the effector functions of tumour-reactive CD8⁺ T cells, favouring mutant outgrowth. Indeed, we demonstrated that *JAK1/2* loss protected melanoma cells from anti-proliferative and pro-apoptotic IFN γ activity. Since *JAK1* is a component also of the type I IFN signalling pathway, an additional selective pressure of IFN α on tumour cells cannot be excluded^{8,31}. In contrast to the *JAK1/2*-deficient melanoma cells, *STAT1* mutant cells were established from a treatment-naive stage III lymph node metastasis suggesting that in this case spontaneous anti-tumour T-cell responses enriched these cells. In addition to melanoma, inactivating mutations in genes related to IFN γ signalling, in particular *JAK1*, have recently been described for microsatellite instable endometrial and colorectal cancers, arguing for a contribution to disease progression also in other malignancies^{32–37}.

In our patient models, *JAK1* deficiency originated from an initial chromosome 1p aberration causing mono-allelic *JAK1* loss in melanoma cells and a subsequent mutation inactivating the remaining *JAK1* allele. Losses of the short arm of chromosome 1 are not uncommon in cutaneous and uveal melanoma with larger deletions occurring in around 10% of cutaneous melanomas^{38,39}. More focal deletions as well as copy number neutral losses of heterozygosity may occur, that we detected at a high frequency in a cohort of 59 melanoma cell lines²¹, showing also a very high frequency of allelic *JAK2* losses (76%). All of these alterations in addition to gene mutations would predispose to *JAK1/JAK2* inactivation and IFN γ -resistance in tumours if put under selective pressure by the immune system.

Interestingly, our data demonstrate that IFN γ -resistant *JAK1/2*-deficient melanoma cells progress to a ‘higher level’ of immunotherapy resistance. We provide evidence for the first time that IFN γ -resistant HLA class I-positive metastases can evolve into HLA class I-negative lesions thereby gaining complete CD8⁺ T-cell resistance. The HLA class I-negative phenotype is caused by a coordinated silencing of genes involved in antigen presentation (*HLA-B*, *HLA-C*, *TAP1*, *TAP2*, *B2M*). Downregulation of this set of genes has previously been reported for melanoma and other tumour entities. The underlying

molecular silencing mechanisms remain unclear but are most likely of epigenetic nature^{40–42}. IFN γ is well known for its role in upregulating antigen processing and presentation thereby augmenting the detection and elimination of malignant cells by tumour antigen-specific CD8⁺ T cells^{43,44}. However, in case of *JAK1/2* deficiency IFN γ -induced restoration of antigen presentation in tumour cells is abrogated. Phenotypically HLA class I-negative *JAK1/2*-deficient metastases share features with tumours lacking HLA class I surface expression due to inactivating *B2M* mutations as described by us and others^{33,45–48}. HLA class I-negative metastases will be resistant towards any type of immunotherapy that is dependent on the activity of HLA class I-restricted tumour antigen-specific CD8⁺ T cells, including adoptive cell therapy and checkpoint modulators. However, in contrast to *B2M* mutants, melanoma cells of the regulatory HLA class I-negative phenotype can regain HLA class I expression to adapt to specific environmental conditions such as metastatic sites (for example, lung, liver) enriched for natural killer cells that are specialized in recognition and killing of HLA class I-negative malignant cells⁴⁹.

Recently, resistance to anti-PD1 and anti-CTLA-4 therapy has been associated with sustained IFN γ signalling upregulating ligands for multiple inhibitory receptors on T cells, as well as IFN γ resistance protecting from cytokine-induced cell cycle arrest/apoptosis^{33–35,50}. It will be of importance to determine how far the alterations we detected in pretreatment biopsies will undergo positive selection in tumours recurring upon anti-PD1 therapy or whether mutations will evolve *de novo* as recently described³³. Of equal importance will be the identification of novel IFN γ -resistance mechanisms. Epigenetic factors as well as altered expression of negative IFN γ pathway regulators in tumour cells or microenvironmental influences could have an additional relevant role in conferring resistance or reduced sensitivity to IFN γ ^{51,52}. Furthermore, the resistance mechanisms could go beyond IFN γ and apply to other cytokines such as TNF α ^{13,14}. In this regards, the combined action of IFN γ and TNF α has been demonstrated to destroy tumour cells and their stroma thereby essentially contributing to the eradication of established mouse tumours⁵³.

It will be a considerable future challenge to identify all mutations associated with IFN γ resistance and to define the coevolution of HLA class I expression in longitudinal melanoma biopsies. Only this strategy will ensure that patients receive the most promising treatment options and be switched to other therapeutic regimens if IFN γ resistance develops, for example, oncogenic pathway inhibitors that could eliminate resistant tumour clones and allow the patients to reinitiate immunotherapy.

Figure 4 | IFN γ -resistant melanoma evolves into a T-cell-resistant lesion. (a) Mutation defined by targeted sequencing on DNA from Ma-Mel-61h cells and autologous blood cells as wild-type (WT) control (ctrl). Plots of aligned sequencing reads in the location where the *JAK1* c.1798G>T, p.G600W mutation was identified. WT sequence shown on the bottom, arrow highlights mutation or corresponding wild-type (WT) site. Number of sequencing reads notated on the left; %, frequency of mutations in reads. (b) Lysates from IFN γ -treated (48 h) Ma-Mel-61b, Ma-Mel-61g and Ma-Mel-61h cells analysed by western blot for expression of IRF1 and HLA class I heavy chains; GAPDH, loading control. Representative data from $n = 3$ independent experiments. (c) HLA class I and CD54 surface expression on IFN γ -treated (48 h) Ma-Mel-61g and Ma-Mel-61h cells, measured by flow cytometry. Representative data from $n = 3$ independent experiments. (d) Immunohistochemical staining of serial cryostat tissue sections from metastasis Ma-Mel-61g for melanoma marker HMB45 and HLA class I. (e) Ma-Mel-61h and Ma-Mel-61g cells, transfected with expression plasmids encoding wild-type *JAK1* or mutant *JAK1*-G600W, analysed for specific mRNA expression by quantitative reverse transcription-PCR in the presence or absence of IFN γ (48 h). Ma-Mel-61h cells served as a control (ctrl). Relative expression levels given as means (\pm s.e.m.) from $n = 2$ independent experiments. (f) HLA class I surface expression on IFN γ -treated (48 h) Ma-Mel-61h-*JAK1* and Ma-Mel-61h-*JAK1*-G600W transfectants, measured by flow cytometry. Representative data from $n = 2$ independent experiments. (g) Ma-Mel-61h and Ma-Mel-61g cells, transfected with expression plasmids encoding wild-type *JAK1* (WT) or mutant *JAK1*-G600W (M), analysed for recognition by autologous CD8⁺ T cells in the presence or absence of IFN γ (48 h). T-cell activation measured as IFN γ release by ELISpot assay. Representative data from $n = 2$ independent experiments.

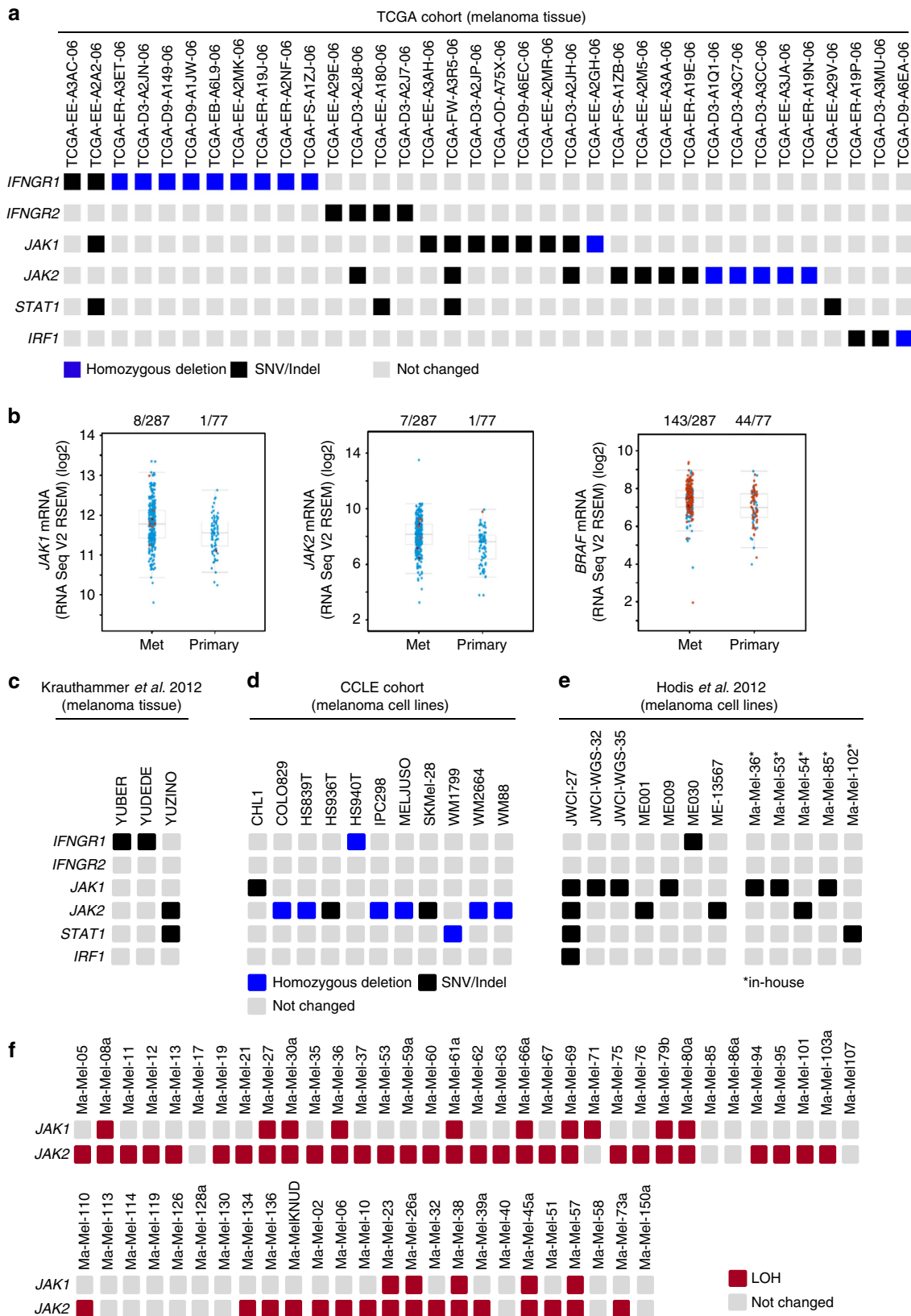


Figure 5 | Mutations in melanoma tissue samples and cell lines. (a) Genetic alterations (homozygous deletions, single-nucleotide variants [SNV], small insertions and deletions [Indel]) in components of the type II IFN signalling pathway in TCGA melanoma samples ($n = 287$, accessed 31/12/2016). (b) Mutations in *JAK1*, *JAK2* and *BRAF* in the TCGA melanoma dataset stratified by tissue origin (primary tumours vs metastases). y axis, indicates expression of corresponding mRNA. Each circle represents a sample, samples harbouring SNV or Indels are shown in red ($n = 287$, accessed 30/12/2016). (c–e) Recurrent mutations of type II IFN signalling pathway genes in published datasets^{15,19,20}. (f) SNP array data from 59 melanoma cell lines²¹ show LOH at the *JAK1* (Chr. 1p31.3) and *JAK2* (Chr. 9p24.1) locus.

Methods

Patients samples. Peripheral blood samples and tumour tissues were collected after written informed patient consent with institutional review board approval. Melanoma cell lines were established from excised metastatic lesions. Cell lines were confirmed to be mycoplasma-free in monthly intervals and authenticated by genetic profiling on genomic DNA at the Institute for Forensic Medicine

(University Hospital Essen) using the AmpFLSTR-Profiler Plus kit (Applied Biosystems). Melanoma cells were cultured in RPMI1640 or DMEM medium with L-glutamine (Gibco/Life technologies) and 10% fetal calf serum. Cells were seeded and rested overnight followed by addition of IFN γ (500 U ml⁻¹, Boehringer Ingelheim) or IFN α 2b (1,000 U ml⁻¹, Essex Pharma) and incubation for indicated periods.

Immunohistochemistry. Serial cryostat tissue sections were stained with antibodies specific for HLA-DR,-DP,-DQ, kindly provided by S. Ferrone⁵⁴, HMB-45 (Dako), HLA class I antigen complexes (W6/32; Dianova) in combination with a Polymer Kit containing an AP-coupled secondary antibody (ZytoChem-Plus AP Polymer Kit, Zytomed).

Isolation of genomic DNA. Five to ten 10 μ m-thick sections of FFPE tissue were deparaffinized according to the following protocol: 2 steps of 10 min xylene, 5 min 100% ethanol, 5 min 95% ethanol, 5 min 70% ethanol, rinsing in water. After drying, tumour tissue was manually macrodissected from the sections. Genomic DNA from tissue as well as 200 μ l of whole blood (normal control) was isolated using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The same kit was used for DNA isolation from pelleted cultured tumour cells, Epstein-Barr virus-transformed B lymphocytes and peripheral blood mononuclear cells.

Targeted sequencing. A custom amplicon-based sequencing panel covering 12 genes of the interferon pathway (Supplementary Table 2) was designed and prepared applying the GeneRead Library Prep Kit from QIAGEN according to the manufacturer's instructions. Individual samples were barcoded using a kit from New England Biosciences and 24 samples sequenced in parallel on an Illumina MiSeq Next Generation Sequencer. Sequencing analysis was performed applying the CLC Cancer Research Workbench from QIAGEN. After trimming the primer sequences, the sequence reads were aligned to the human genome assembly 19 (hg19). Analysis for both in/dels and SNVs followed. SNPs were filtered out by cross-referencing the dbSNP database, the 1,000 genomes database and in individual cases manually. Mutations affecting the coding region of the gene were considered if predicted to result in non-synonymous amino acid changes, overall coverage of the mutation site was ≥ 30 reads, > 5 reads reported the mutation variant and the frequency of mutated to unmutated reads was $\geq 10\%$.

SNP array analysis. SNP arrays were performed using the CytoScan HD Array from Affymetrix. Hybridization was done according to the manufacturer's protocol and data analysis performed applying the program Chromosome Analysis Suite from Affymetrix.

Quantitative real-time PCR. Total mRNA was isolated from tumour cells using the RNeasy plus Mini Kit (Qiagen), in combination with RNase-free DNase Set (Qiagen) according to the manufacturer's instructions. Reverse transcription, TaqMan-based real-time PCR and calculation of relative expression were performed as described previously⁵⁵. Taqman assay systems specific for *HLA-B*, *HLA-C*, *TAP1*, *TAP2*, *B2M*, *GAPDH* were purchased from Thermo Fisher. In all experiments the amount of specific mRNA was normalized to endogenous *GAPDH* mRNA levels.

Analyses of published melanoma data sets. Kaplan–Meier survival plots, log-rank tests and multivariate Cox-regressions based on differentially expressed genes from TCGA melanoma samples were assessed using the UZH cancer browser samples⁵⁶, using the 10% percentile for comparing samples with the lowest and the highest mRNA expression for *IFNG*, *STAT1*, *IRF1* and *CD8A*. Information on mutations, including single-nucleotide variants (SNV), small insertions/deletions (Indels) and copy number variants (considering only homozygous deletions) in genes of the type II IFN signalling pathway (*IFNGR1*, *IFNGR2*, *JAK1*, *JAK2*, *STAT1*, *IRF1*) were obtained from TCGA skin cutaneous melanoma (SKCM) samples with complete mutation data ($n = 287$, accessed 31/12/2016). Survival analyses based on mutations and aberrant protein expression were based on the extended SKCM TCGA data set ($n = 487$) and clinical data obtained from cBioPortal, generating

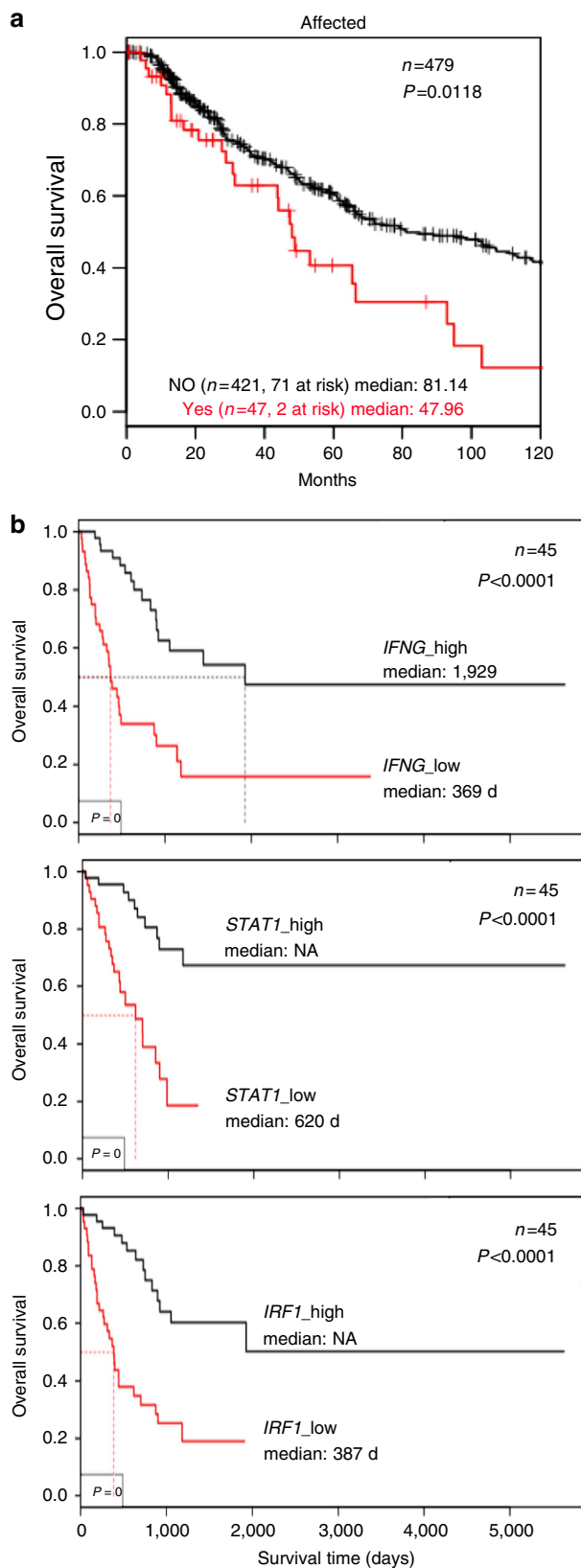


Figure 6 | Alterations in IFN γ pathway signalling impact on patient survival. (a) Truncating mutations, homozygous deletions and low protein levels of *IFNGR1*, *IFNGR2*, *JAK1*, *JAK2*, *STAT1* and *IRF1* define a subset of melanoma patients in the TCGA data set with decreased survival. Of 479 patients with data on aberrations, 468 had available survival data. Log-rank P value shown. (b) Kaplan–Meier survival curves for *IFNG*, *STAT1* and *IRF1* expressing TCGA melanomas⁵⁶, P values shown from Log-rank tests. Multivariate P values corrected for age: *IFNG* $P = 1.79 \times 10^{-5}$, $n = 90$, 47 events; *STAT1* $P = 3.82 \times 10^{-5}$, $n = 90$, 33 events; *IRF1* $P = 2.52 \times 10^{-5}$, $n = 90$, 44 events.

Table 2 | Mutations in biopsies from patients receiving anti-PD1 (n = 59) and anti-CTLA4 (n = 110) immunotherapy.

Material	Sample	Gene	AA change	cDNA change	Treatment	Response
Patient	15-12774	<i>IRF1</i>	W195*	584G > A	Nivo	PD
Patient	D5923-13	<i>JAK2</i>	D710fs	2123_2124insC	Pemb	PD
Patient	31968-14	<i>JAK1</i>	R681Q	2042G > A	Pemb	PD
Patient	14-28049	<i>JAK1</i>	E501K	1501G > A	Nivo	CR
Patient	D788-15	<i>JAK1</i>	E903K	2707G > A	Nivo	PD
Patient	13-12191	<i>JAK2</i>	H103Y	307C > T	Nivo	PR
Patient	32616-13	<i>JAK2</i>	S15F	44C > T	Nivo	PR
Patient	18298-15	<i>JAK2</i>	T376fs	1125delA	Pemb	PR
Patient	5870-14 [†]	<i>JAK2</i>	P121S	361C > T	Nivo	SD
Patient	D2788-15	<i>JAK1</i>	K249N	747G > T	Nivo	Unknown
Patient [‡]	343-16 [§]	<i>JAK1</i>	L149F, T147I	445C > T, 440C > T	Nivo	PD
Patient	132	<i>IFNGR1</i>	D465N	1393G > A	Ipilimumab	PR
Patient	4	<i>JAK1</i>	R110_splice	e5-1	Ipilimumab	PR
Patient	14	<i>JAK1</i>	G590R	1768G > C	Ipilimumab	PD
Patient	151	<i>JAK1</i>	V715M	2143G > A	Ipilimumab	PD
Patient	151	<i>JAK1</i>	G655D	1964G > A	Ipilimumab	PD
Patient	37	<i>JAK1</i>	G182E	545G > A	Ipilimumab	PD
Patient	4	<i>JAK2</i>	R138Q	413G > A	Ipilimumab	PR
Patient	163	<i>JAK2</i>	G164A	491G > C	Ipilimumab	PD
Patient	110	<i>IRF1</i>	R314W	940C > T	Ipilimumab	PD
Patient	82	<i>IRF1</i>	S221F	662C > T	Ipilimumab	PD

AA, amino acid; cDNA, complementary DNA; CR, complete response; fs, frameshift; ins, insertion; Nivo, Nivolumab; PD, progressive disease; Pemb, pembrolizumab; PR, partial response; SD, stable disease; splice, splice site mutation.

*Nonsense—stop codon mutation.

[†]Additionally a *IFNGR1*, E195K, 685G > A mutation.

[‡]Relapse under treatment.

[§]Additionally a *IFNGR1*, G129E, 461G > A and *IFNGR2*, T187I, 503C > T mutation.

Kaplan–Meier survival plots and log-rank tests in R (R Development Core Team; <http://www.R-project.org>). Coexpression plots were obtained for TCGA SKCM samples using cBioPortal⁵⁷. Mutation calls, including SNV and Indels, from 110 patients before anti-CTLA-4 antibody therapy were reported previously⁷. Additional mutation data for melanoma tissues and cell lines with SNV/Indels^{15,20} as well as for melanoma cell lines including also homozygous deletions, as part of the CCLE project¹⁹ were assessed using cBioPortal⁵⁷.

SNP array data from 59 melanoma cell lines analysed with the 250 k StyI SNP array of the Affymetrix GeneChipV 500 K array set (Affymetrix, Santa Clara, CA), GEO accession number GSE17534 (ref. 21) were assessed for LOH using Affymetrix genotyping console software. In 44 cases, SNP data from corresponding germline DNA were available for paired analysis. In the remaining cases, the SNP data from tumour samples were compared with baseline values obtained from combined analysis of the SNP data from the 44 available germline cases.

Western blot. Proteins from tumour cell lysates were separated by SDS–polyacrylamide gel electrophoresis, blotted on nitrocellulose membranes and probed with the following primary antibodies: anti-STAT1 (Santa Cruz, clone M-22, 1:1,000) and anti-pSTAT1 (Cell Signaling, clone 58D6, 1:1,000), anti-IRF1 (Santa Cruz, clone H-205, 1:500) and anti-GAPDH (Cell Signaling, 14C10, 1:5,000). HC10 (1:1,000) was used for detection of β 2m-free HLA heavy chains^{58,59}. After washing, membranes were incubated with the appropriate secondary antibodies linked to horseradish peroxidase. Antibody binding was visualized with the enhanced chemiluminescence (ECL) system. Full scans of western blots are depicted in Supplementary Figs 7–9.

Flow cytometry. The following directly labelled antibodies were used for staining of cellular surface markers: anti-HLA-ABC-APC (eBiosciences, clone W6/32; 1 μ l), anti-CD54-PE (Beckmann Coulter, clone 84H10; 2.5 μ l), anti-PD-L1-PE (Biolegend, clone 29E2A3; 5 μ l) and anti-HLA-DR-PC7 (Beckmann Coulter, clone Immun-357; 2.5 μ l). After fixation, stained cells were analysed by flow cytometry on a Gallios flow cytometer (Beckmann Coulter) and Kaluza (Beckman Coulter) software, respectively, for data analysis. In order to isolate specific Ma-Mel-36 subpopulations, cells were stained with anti-HLA-DR-PC7 and sorted based on the specific expression of the surface markers by flow cytometry on an Aria II cell sorter and the FACS Diva software (BD Biosciences).

Plasmid generation and transfection. Wild-type *JAK1* was amplified using Phusion High-Fidelity DNA Polymerase (NEB) and the following primers: *JAK1*-SPAfo: 5'-ATCGTCTCGAGATGCAGTATCTAAATATAAAA-3' and *JAK1*-SPARe: 5'-ATTGCTCATATGTTTTAAAAGTCTTCAAATCC-3'. After restriction digest with *XhoI* and *NdeI*, the PCR product was ligated into the PMZ3F vector (kindly provided by the laboratory of Jack Greenblatt, University of

Toronto)⁶⁰. Protein expression was verified by immunoblotting using a Flag-specific antibody (Sigma). The point mutation was introduced using Quikchange mutagenesis (Agilent) according to the manufacturer's protocol and the following primers: *JAK1*G600Wfo: 5'-ACACACATCTATTCTTGGACCCCTGA TGGATTA-3' and *JAK1*G600Wre: 5'-TAATCCATCAGGGTCCAAGAAATAGAT GTGTGT-3'. The intended point mutations were verified by DNA sequencing and protein expression was examined by immune blotting. Lipofectamine (Life Technologies) was used for plasmid transfection of melanoma cells. After 48 h, cells were harvested and subjected to further analyses or treated with G418 for enrichment of transfectants.

Real-time proliferation assay (xCELLigence). For background measurement 50 μ l medium was added to an E-Plate 96 (Roche). Subsequently, melanoma cells were seeded in an additional volume of 100 μ l medium. Cell attachment was monitored using the RTCA SP (Roche) instrument and the RTCA software Version 1.1 (Roche). After 20–24 h cells were treated with IFN γ (500 U ml⁻¹) or left untreated, followed by incubation for 7 d at 37 °C. All experiments were performed in duplicates. Changes in electrical impedance were expressed as a dimensionless cell index value, which derives from relative impedance changes corresponding to cellular coverage of the electrode sensors, normalized to baseline impedance values with medium only.

Expansion of autologous tumour-reactive T cells. Tumour-reactive T cells were expanded following a previously described protocol⁴⁷. Briefly, CD8⁺ T lymphocytes were isolated from cryopreserved peripheral blood mononuclear cells using anti-CD8 MicroBeads (Miltenyi Biotec). Isolated T cells (1 \times 10⁶) were co-cultured in 24-well culture plates with 1 \times 10⁵ irradiated (100 Gy or 120 Gy) autologous tumour cells per well in 2 ml of AIM-V (GIBCO/BRL) supplemented with 10% (vol/vol) human AB serum. Medium was supplemented with IL-2 (250 U ml⁻¹) on day 3. CD8⁺ T cells were restimulated at weekly intervals with irradiated melanoma cells. After two rounds of restimulation, T cells were subjected to ELISpot assays.

IFN γ ELISpot assay. IFN γ enzyme-linked immunospot (ELISpot) assay was performed as previously described⁶¹. Briefly, multiscreen-HA plates (Millipore, Bedford, MA) were coated with 5 μ g ml⁻¹ anti-hIFN γ -mAb 1-D1K (Mabtech). T cells were seeded in RPMI medium and added at indicated numbers to 1 \times 10⁴ tumour cells per well. After 20–24 h incubation at 37 °C in 5% CO₂, a biotinylated secondary anti-hIFN γ antibody (1 μ g ml⁻¹, clone 7-B6-1, Mabtech) was added and spots were developed by sequential addition of 1:1,000 diluted Extravidin alkaline phosphatase and BCIP/NBT Liquid Substrate System (Sigma-Aldrich). Spot numbers were determined with the AID ELISpot reader (AID Diagnostika).

Data availability. SNP array data files are accessible at the NCBI GEO database. Cell lines used in this study can be obtained by MTA.

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Author contributions

A.S. established patient models, performed targeted sequencing, analysed data; F.Z. carried out T-cell assays; C.H. performed T-cell assays and IHC; N.P. performed western blots, PCR, apoptosis and proliferation assays; R.M. carried out western blots, transfections, proliferation assays; N.S. supported targeted sequencing; B.R. helped with western blots; N.B. carried out IHC; Se.H. generated expression plasmids; B.W., R.G., J.U., C.L. and H.G. provided patient samples; L.K.-H. and M.Z. performed SNP arrays and data analyses; A.M.W. carried out cell sorting; M.T. supported plasmid constructions, edited the manuscript; Su.H. performed bioinformatics; B.S. collected patient samples, edited the manuscript; D.S. established patient models, contributed to conception and design of the study; K.G. contributed to conception and design of the study, carried out data analyses, edited manuscript; A.P. designed experiments, analysed data, wrote manuscript.

Additional Information

Accession code: SNP array data files are accessible at the NCBI GEO database under GSE96884.

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