



HAL
open science

Adipocyte Extracellular Vesicles Decrease p16INK4A in Melanoma: An Additional Link between Obesity and Cancer

Ikrane Lazar, Emily Clement, Lorry Carrié, David Esteve, Stéphanie Dauvillier, Mohamed Moutahir, Stéphane Dalle, Véronique Delmas, Nathalie Andrieu-Abadie, Lionel Larue, et al.

► To cite this version:

Ikrane Lazar, Emily Clement, Lorry Carrié, David Esteve, Stéphanie Dauvillier, et al.. Adipocyte Extracellular Vesicles Decrease p16INK4A in Melanoma: An Additional Link between Obesity and Cancer. *Journal of Investigative Dermatology*, 2022, 142 (9), pp.2488-2498.e8. 10.1016/j.jid.2022.01.026 . hal-04246204

HAL Id: hal-04246204

<https://hal.science/hal-04246204>

Submitted on 17 Oct 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Adipocyte extracellular vesicles decrease p16^{INK4A} in melanoma: a new link between obesity and cancer

Ikrame Lazar^{*1}, Emily Clement^{*1}, Lorry Carrié^{*1,6}, David Esteve¹,
Stéphanie Dauvillier¹, Mohamed Moutahir¹, Stéphane Dalle², Véronique Delmas³⁻⁵,
Nathalie Andrieu-Abadie⁶, Lionel Larue^{#3-5}, Catherine Muller^{#1,5} and Laurence Nieto^{§1,6}

1- Institut de Pharmacologie et de Biologie Structurale (IPBS) UMR 5089, Université de Toulouse, CNRS, UPS, Toulouse, 31077, France

2- Department of Dermatology, Centre Hospitalier Lyon Sud, Pierre Bénite Cedex, 69495, France

3- Institut Curie, Université PSL, CNRS UMR3347, Inserm U1021, Normal and Pathological Development of Melanocytes, Orsay, 91400, France

4- Université Paris-Saclay, CNRS UMR3347, Inserm U1021, Signalisation radiobiologie et cancer, 91400 Orsay, France

5- Equipe Labellisée Ligue Contre le Cancer

6- Université Fédérale de Toulouse Midi-Pyrénées, Université Toulouse III Paul-Sabatier, Inserm, Centre de Recherches en Cancérologie de Toulouse UMR 1037, Toulouse, 31037, France.

*/#: these authors contributed equally to this work

§ Corresponding author: Pr Laurence Nieto.

Present address: Centre de Recherches en Cancérologie de Toulouse, Inserm UMR1037, Oncopole de Toulouse, 2 avenue Hubert Curien CS 53717, Toulouse cedex 1 31037, France. Phone: (33) 582741618; Fax: (33) 582741685; @mail: laurence.nieto@inserm.fr

ORCID

Ikrame Lazar: 0000-0003-4062-3627; Emily Clement: 0000-0001-6027-6488; Lorry Carrié: 0000-0003-1792-136X; David Esteve: 0000-0002-7359-0326, Stéphanie Dauvillier: , Mohamed Moutahir: , Stéphane Dalle: 0000-0002-3423-4548; Véronique Delmas: 0000-0001-7368-3664; Nathalie Andrieu-Abadie: 0000-0003-2698-1970; Lionel Larue: 0000-0002-2010-6107 ; Catherine Muller: 0000-0003-2282-9322; Laurence Nieto: 0000-0002-1006-0168

RUNNING TITLE

p16^{INK4A} links melanoma and obesity

ABBREVIATIONS

AT: adipose tissue; Ad-CM: adipocyte-conditioned medium; Ad-EV: adipocyte extracellular vesicles; HFD: high fat diet; ND: normal diet; GEMM: genetically engineered mouse models;

3470 words / 3500

ABSTRACT

Obesity is a recognized factor for increased risk and poor prognosis of many cancers, including melanoma. Here, using genetically engineered mouse models of melanoma (NRAS^{Q61K} transgenic expression, associated or not with *Cdkn2A* heterozygous deletion), we show that obesity increases melanoma initiation and progression by supporting tumor growth and metastasis thereby reducing survival. This effect is associated with a decrease in p16^{INK4A} expression in tumors. Mechanistically, adipocytes downregulate p16^{INK4A} in melanoma cells through β -catenin-dependent regulation, which increases cell motility. Furthermore, β -catenin is directly transferred from adipocytes to melanoma cells in extracellular vesicles, thus increasing its level and activity, which represses p16^{INK4A} transcription. Adipocytes from obese individuals have a stronger effect than those from lean individuals, mainly due to an increase in the number of vesicles secreted, thus increasing the amount of β -catenin delivered to melanoma cells, and, consequently, amplifying their effect. In conclusion, here, we reveal that adipocyte extracellular vesicles control p16^{INK4A} expression in melanoma, which promotes tumor progression. This work expands our understanding of the cooperation between adipocytes and tumors, particularly in obesity.

INTRODUCTION

According to the World Health Organization, a third of the world's population is obese or overweight, making excess body fat a major public health issue, particularly due to comorbidities such as cardiovascular diseases, diabetes and cancer. Obesity, defined by excessive adipose tissue (AT) accumulation, leads to alterations in AT metabolism, secretions and inflammatory status (Longo et al., 2019). These changes mediate local and systemic effects, contributing to the deleterious impact of obesity on tumor incidence and progression (Park et al., 2014). Indeed, obesity increases both the incidence and the aggressiveness of many cancers (Park et al., 2014; Renehan et al., 2008). Adipocytes, the main component of AT, play a key role in tumor progression by fueling cancer cell metabolism with lipids, secreting pro-tumoral soluble factors such as chemokines, pro-inflammatory cytokines and hormones, and remodeling the extracellular matrix (Andarawewa et al., 2005; Dirat et al., 2011; Iyengar et al., 2005; Laurent et al., 2016; Nieman et al., 2011). Among signals emanating from adipocytes, we have shown that adipocyte extracellular vesicles (Ad-EV) convey a multitude of molecules to tumor cells, including the enzymes and substrates required to fuel fatty acid metabolism, promoting aggressiveness (Clement et al., 2020; Lazar et al., 2016). In obesity, the impact of adipocytes on tumor progression increases (Duong et al., 2017), due to changes in their secretions, including their EV (Le Lay et al., 2021; Ouchi et al., 2011). However, many of the molecular mechanisms associated remain elusive.

Cutaneous melanoma, here referred to as 'melanoma', is a skin cancer derived from melanocytes that can become invasive, infiltrate surrounding tissues including subcutaneous AT, and ultimately metastasize to other organs. In such cases, despite major treatment improvements, survival rates remain poor. Thus, determining the factors that contribute to melanoma progression is crucial to discern new therapeutic targets. Epidemiological data show that obesity adversely impacts melanoma incidence and progression (Fang et al., 2017;

Sergentanis et al., 2013; Skowron et al., 2015; Stenehjem et al., 2018). The deleterious association between obesity and melanoma progression has been confirmed in murine melanoma models in which cell lines are grafted in animals fed a high fat diet (HFD) (Malvi et al., 2015) or genetically modified (Brandon et al., 2009) to induce obesity. In these studies, a positive correlation between obesity and melanoma size, lymph node involvement and lung metastasis have been observed [reviewed in (Clement et al., 2017)]. Nevertheless, these grafted models fail to recapitulate the physiopathological processes of melanomagenesis. Therefore, genetically engineered mouse models (GEMM) would be more appropriate to fully understand the impact of obesity on melanoma.

Here, in two GEMM that spontaneously develop melanoma, we show that obesity increases melanoma initiation and progression, and is associated with a decrease in p16^{INK4A} tumor expression. Adipocytes orchestrate p16^{INK4A} downregulation in melanoma cells, promoting aggressiveness *in vitro*. Mechanistically, Ad-EV transport β -catenin to melanoma cells, increasing its level and activation, which ultimately results in the repression of p16^{INK4A} transcription in recipient cells. In obesity, adipocytes secrete more EV, increasing β -catenin levels even further, thus amplifying their pro-tumoral effect.

RESULTS

Obesity increases melanoma incidence and aggressiveness and downregulates p16^{INK4A} in tumors

The two GEMM used in this study are described in [Figure 1a](#). The first model, referred to as NRAS, carries the Q61K-mutated NRAS protein, found in 28% of human skin melanomas (Cancer Genome Atlas Network, 2015), specifically expressed in melanocytes. The mutant protein is constitutively active, resulting in the activation of the MAPK and PI3K-AKT pathways. These mice develop melanomas with a low penetrance and a high latency

(Ackermann et al., 2005). In the second model (NRAS-p16), NRAS mice were crossed with mice lacking the *Cdkn2a* locus, encoding p16^{INK4A} and p19^{ARF} to bypass senescence (de Snoo and Hayward, 2005). Mono and bi-allelic deletion of this locus accelerates melanoma initiation and progression (Ackermann et al., 2005). To appropriately evaluate the influence of obesity on tumor development, we used heterozygous animals whose melanoma development is intermediate compared to their WT and total KO counterparts (Ackermann et al., 2005). In crossbred animals (NRAS-p16), melanomas develop with a greater penetrance and a reduced latency compared to NRAS mice (Ackermann et al., 2005). Importantly, in patients, NRAS^{Q61K} mutations and *CDKN2A* deletions are frequently associated (Cancer Genome Atlas Network, 2015; Hayward et al., 2017), resulting in aggressive tumors (Boespflug et al., 2017; Jakob et al., 2012).

In NRAS mice fed a HFD to induce obesity, the latency for melanoma development is reduced and penetrance is increased (Figure 1b). Moreover, in obesity, melanomas were more aggressive, with more tumors per animal, faster tumor growth, increased lymph node size and a higher proportion of animals bearing metastases (Figure 1c-f). Obesity had a similar effect in the heterozygous NRAS-p16 model with reduced tumor latency and increased aggressiveness when considering the same parameters (Figure 1b-f).

Strikingly, the survival curve of HFD-fed NRAS mice almost perfectly superimposes that of normal diet (ND)-fed NRAS-p16 mice (Figure 1b), and tumor growth and metastasis were also similar in these two groups (Figure 1c-f), suggesting that obesity may mimic the effects of p16^{INK4A} loss, increasing melanoma aggressiveness.

To test this hypothesis, we labeled p16^{INK4A} protein in melanoma isolated from mice fed a ND or a HFD. First, in ND mice, as expected, we observed that p16^{INK4A} levels are reduced in NRAS-p16 animals compared to NRAS, although the protein is still present as these mice bear only a heterozygous *CDKN2A* deletion. Consistent with our hypothesis, p16^{INK4A} levels are

decreased in melanoma from obese mice compared to their lean counterparts. Moreover, results were again similar between melanoma from NRAS mice fed a HFD and NRAS-p16 fed a normal one (Figure 2).

Adipocytes decrease p16^{INK4A} expression in melanoma cells, a process amplified in obesity

As our results showed that melanoma aggressiveness is increased in obesity, with a concomitant decrease in p16^{INK4A} levels, and adipocytes are known to orchestrate many processes linking obesity and tumor progression due to modifications in their phenotype and secretory profile (Duong et al., 2017), we hypothesized that adipocytes may be responsible for p16^{INK4A} downregulation in melanoma cells, increasing tumor progression. Indeed, in addition to its role in senescence bypass, p16^{INK4A} protein has been associated with melanoma invasion and metastasis (Reed et al., 1995; Ruiz et al., 1998; Zeng et al., 2018).

We tested this hypothesis by using two independent approaches. First, as primary adipocytes cannot be maintained in culture for more than 24 hours (Lafontan, 2012), we developed a 3D system in which these cells isolated from lean or obese individuals were embedded in a matrix. In this system, adipocytes remain healthy for at least 5 days, allowing us to perform co-culture with melanoma cells (Figure S1). Using this 3D co-culture assay with the BRAF^{V600E}-mutated human SKMEL28 melanoma cell line, we showed that human adipocytes increase melanoma cell motility with a stronger effect in obesity (Figure 3a, left), consistent with previous findings (Clement et al., 2020; Lazar et al., 2016). Moreover, in accordance with our hypothesis, the level of p16^{INK4A} is decreased in melanoma cells co-cultivated with adipocytes, and is further reduced when these adipocytes were isolated from obese individuals (Figure 3a, right). Similar results were obtained in a second approach, in which SKMEL28 cells were treated with human adipocyte-conditioned medium (Ad-CM) (Figure 3b), indicating that adipocyte-derived secretions mediate changes in p16^{INK4A} expression. These results were confirmed with other

human melanoma cell lines, with different mutational statuses as well as with 1014 melanoma cells, a murine allogenic cell line derived from the NRAS mouse model (Petit et al., 2019) that were co-cultivated with murine adipocytes or treated with murine Ad-CM. When treated with Ad-CM, motility increased and p16^{INK4A} expression decreased in all cell lines tested, with a heightened effect with adipocytes from obese individuals (Figure S2a-b), suggesting that these effects are not dependent on mutational status, nor species.

Adipocyte control of p16^{INK4A} expression occurs at the transcriptional level, as mRNA levels decreased after co-culture or Ad-CM treatment, with a stronger effect in obesity (Figure 3c and Figure S2c).

Finally, to validate the link between p16^{INK4A} downregulation and tumor aggressiveness in our system, we knocked-down p16^{INK4A} in melanoma cells, using two independent siRNA. In accordance with a previous study (Zeng et al., 2018), p16^{INK4A} silencing promotes melanoma cell motility (Figure 3d). In cells treated with Ad-CM, p16^{INK4A} is decreased by adipocyte secretions (Figure 3b and Figure S2b). Consequently, p16^{INK4A} silencing has a much lesser effect on motility in cells treated with lean Ad-CM, compared to control cells, and no effect on those treated with obese Ad-CM (Figure 3d), attesting that the decrease in p16^{INK4A} induced by adipocytes is responsible for their effect on melanoma cell motility (shown in Figure 3b and Figure S2a).

Altogether, these data demonstrate that adipocytes decrease p16^{INK4A} expression in melanoma cells, which promotes their migration. In obesity, p16^{INK4A} levels are decreased even further and melanoma aggressiveness is heightened.

Obesity increases adipocyte-dependent β -catenin content and activity in melanoma cells

We have previously shown that β -catenin (CTNNB1) can repress p16^{INK4A} transcription in melanoma cells by a direct control of *CDKN2A* promoter (Delmas et al., 2007). Therefore, we

hypothesized that this process may be responsible for adipocyte-mediated downregulation of p16^{INK4A} in melanoma cells. Accordingly, following co-culture with adipocytes or treatment with Ad-CM, β -catenin accumulates in the nucleus of melanoma cells, as opposed to non-treated melanoma cells in which it is predominantly localized in the cytoplasm or at the plasma membrane. This phenomenon is exacerbated with adipocytes collected from obese individuals (Figure 4a and Figure S3). The activity of nuclear β -catenin is largely mediated by the TCF/LEF transcription factors, including p16^{INK4A} transcriptional repression in melanoma (Delmas et al., 2007), and we postulated that such a process could be involved here. Accordingly, using a top flash assay in which TCF/LEF cis-regulatory sequences control luciferase expression, we observed that luciferase activity was increased when melanoma cells were treated with Ad-CM, validating that adipocytes increase β -catenin transcriptional activity, this process being amplified in obesity (Figure 4b). Moreover, using two different β -catenin inhibitors that both inhibit β -catenin activity by blocking its interaction with TCF/LEF, we confirmed, in control cells, that β -catenin activation represses p16^{INK4A} expression as both inhibitors increased p16^{INK4A} proteins levels in melanoma cells. Importantly, this inhibition reversed the decrease in p16^{INK4A} levels induced by adipocytes (Figure S4a). Collectively, these results show that adipocytes increase β -catenin activation, which acts as a repressor on the *CDKN2A* promoter. Finally, not only is β -catenin redistributed to the nucleus in melanoma cells treated with Ad-CM but its overall protein level is also increased, with a stronger effect in obesity, whereas β -catenin mRNA content remains unchanged (Figure 4c-d).

Therefore, adipocytes increase β -catenin protein in melanoma cells without affecting its mRNA levels and induce its nuclear redistribution and activation, leading to p16^{INK4A} transcriptional repression, a process amplified by obesity.

Ad-EV drive β -catenin-dependent p16^{INK4A} repression

As adipocytes increase β -catenin protein but not its mRNA, we investigated whether β -catenin mRNA depletion by siRNA in melanoma cells could affect adipocyte-induced p16^{INK4A} regulation. In untreated cells, β -catenin silencing slightly increases p16^{INK4A}, but not in cells exposed to Ad-CM (Figure S4b). On the contrary, as shown above, blocking the transcriptional activity of β -catenin increases p16^{INK4A} protein content in melanoma cells, whether they were treated, or not, with Ad-CM (Figure S4a).

Collectively, these results show that i) adipocytes increase β -catenin protein but not its mRNA, ii) p16^{INK4A} downregulation in melanoma cells does not depend on β -catenin mRNA levels but depends on its activity. In light of these results, and other data previously published by our group (Clement et al., 2020; Lazar et al., 2016), we hypothesized that adipocytes directly provide melanoma cells with β -catenin protein, more precisely within EV. Indeed, we have previously shown that β -catenin is present in murine Ad-EV (Clement et al., 2020; Lazar et al., 2016). Moreover, using a stable isotope labeling of amino acids in cell culture (SILAC)-based mass spectrometry assay, we demonstrated that β -catenin is transferred from adipocytes to melanoma cells *via* Ad-EV (Clement et al., 2020). In accordance, Ad-EV increase β -catenin protein levels, induce its nuclear redistribution and decrease p16^{INK4A} mRNA and protein levels in melanoma cells, whereas EV-depleted Ad-CM, here referred to as soluble factors, had no effect (Figure 5). To further demonstrate the role of adipocyte-derived β -catenin in this process, adipocytes were treated with JW74, a tankyrase inhibitor inducing β -catenin degradation through AXIN2 stabilization (PMID: 21199802). JW74 treatment resulted in the secretion of Ad-EV exhibiting a strong decrease in β -catenin protein level (Figure S5a). Moreover, β -catenin-depleted Ad-EV lost their pro-migratory effect on melanoma cells. Therefore, the effect of Ad-EV is dependent on β -catenin transfer from adipocytes to melanoma cells (Figure S5b-c).

We also observed that human adipocytes secrete more EV in obesity compared to lean conditions (Figure 6a). The amount of β -catenin in the EV secreted by adipocytes in obesity is increased when compared to the EV secreted by an equal number of lean adipocytes (Figure 6b). However, β -catenin level is not significantly different when comparing an equal number of Ad-EV secreted in lean or obese conditions (Figure S6a), indicating that the rise is mainly due to an increase in the number of vesicles secreted in obesity rather than the amount of β -catenin per vesicle. Accordingly, melanoma cells treated with an equal number of Ad-EV isolated from lean or obese individuals exhibited similar behavior, concerning p16^{INK4A} and β -catenin levels and β -catenin nuclear localization (Figure S6b-c). Although, when melanoma cells were treated with EV secreted by an equal number of adipocytes, β -catenin protein increases further and its nuclear redistribution is enhanced with obese Ad-EV, whereas p16^{INK4A} levels are decreased when compared to lean Ad-EV (Figure 6c-d).

In conclusion, Ad-EV convey β -catenin protein to melanoma cells. Once transferred, β -catenin accumulates in the nucleus where it inhibits *CDKN2A* expression, decreasing p16^{INK4A} protein and, consequently increasing melanoma cell motility. In obesity, all these processes were amplified, mainly due to an increased number of EV released by adipocytes.

DISCUSSION

Our results show that adipocytes induce p16^{INK4A} downregulation, promoting melanoma cell motility, a key cancer hallmark, and this process is amplified in obesity. Although 45% of melanomas present a downregulation of *CDKN2A* that occurs by genetic alterations or promoter methylation (Cancer Genome Atlas Network, 2015; Hayward et al., 2017; Hodis et al., 2012; Ming et al., 2020), in less aggressive cases, defined by their lower propensity to invade, p16^{INK4A} is still present, at varying levels (Ming et al., 2020; Reed et al., 1995; Shain et al.,

2015; Zeng et al., 2018). In such tumors, obesity could further decrease p16^{INK4A} expression, worsening the patient's prognosis.

It is well known that p16^{INK4A} is a tumor suppressor, involved in senescence bypass, which contributes to melanoma initiation (Ackermann et al., 2005; Dhomen et al., 2009; Krimpenfort et al., 2001). However, loss of *CDKN2A* promotes not only melanoma formation, but also metastases (Reed et al., 1995; Shain et al., 2015; Talve et al., 1997; Zeng et al., 2018), the crucial step in melanoma progression that leads to fatal complications in patients. Despite these studies, the mechanisms linking *CDKN2A* downregulation to invasive behavior is poorly characterized. Recently, Zeng et al showed that bi-allelic loss of p16^{INK4A} enhances melanoma cell invasion, through increased BRN2 expression, a transcription factor associated with melanoma progression (Zeng et al., 2018). Future studies should investigate the mechanisms linking adipocyte-induced p16^{INK4A} reduction and melanoma aggressiveness, with particular focus on the role of BRN2 in lean and obese individuals.

Although the present work focuses on the role of p16^{INK4A} in tumor progression and was performed in malignant cells already immortalized, as stated above, this protein is a well-known tumor suppressor, whose loss can provoke melanomagenesis by inducing senescence bypass (Ackermann et al., 2005; Dhomen et al., 2009; Krimpenfort et al., 2001). Results obtained in our GEMM suggest that adipocyte-dependent p16^{INK4A} downregulation may also contribute to melanoma initiation (Figure 1b), a finding that, although beyond the scope of our current study, merits investigation in the future, particularly as epidemiological data points towards an increased risk of developing melanoma in obese individuals (Clement et al., 2017; Sargentanis et al., 2013).

Here, we have shown that β -catenin-dependent p16^{INK4A} downregulation depends on Ad-EV. Previous data reported that Wnt/ β -catenin signaling pathways in recipient cells could be activated by EV, through the transfer of Wnt proteins, miRNAs or by a direct transfer of

activated β -catenin (Koni et al., 2020). Nevertheless, we, and others, have not identified Wnt proteins in adipocyte EV by mass spectrometry (reviewed in PMID: 33316953, PMID: 33670146). Moreover, our results are in accordance with the latter hypothesis as β -catenin is transferred from adipocytes to melanoma cells in EV and our proteomic studies are in accordance with an active form of the protein in Ad-EV (Clement et al., 2020) and here, we have shown that Ad-EV depleted of β -catenin lose their effect on p16^{INK4A} expression in melanoma cells. However, other proteins and/or RNA, present in Ad-EV, may also contribute to its activation.

We have previously shown that Ad-EV also provide melanoma cells with the substrate and the protein machinery required to carry out fatty acid oxidation and that this metabolic rewiring increases aggressiveness, a process exacerbated in obesity (Clement et al., 2020). Therefore, Ad-EV may influence melanoma cells *via* different mechanisms, with a particularly strong effect in obesity, underlining the importance of these vesicles in melanoma progression. Nevertheless, we cannot exclude that these two processes could cooperate, as studies have shown that p16^{INK4A} and β -catenin can also affect fatty acid oxidation (Deleye et al., 2020; Senni et al., 2019).

The prevalence of aberrations involving the p16^{INK4A}-cyclin D1-CDK4/6-Rb pathway make it an attractive therapeutic target to reduce melanoma aggressiveness. The present study shows that targeting this pathway could be especially relevant for obese patients.

MATERIALS AND METHODS

Materials, including antibodies references and primer sequences, and methods for western blot, RT-qPCR, immunofluorescence, motility and reporter gene assay are provided in supplementary materials and methods (supM&M).

Murine models, tumor collection and histology

The transgenic Tyr::NRAS^{Q61K/0} and Tyr::NRAS^{Q61K/0}; Ink4A-Arf^{+/-} mouse lines (Sohier et al., 2018) were handled in accordance with French and European Union laws (Authorization number #5586-2016060714128573).

Mice fed a ND and HFD, as described in suppM&M, were weighed and examined for tumor appearance and size weekly. Once tumors reached 1 cm in diameter, or if mice were in too poor health, they were euthanized and autopsied. To measure tumor and lymph node size in the GEMM, the volume of the largest melanoma or inguinal lymph node was used.

Melanomas were embedded in paraffin, processed and analyzed by immunohistochemistry as described in suppM&M.

Primary adipocyte isolation and CM preparation

For murine AT isolation, inguinal subcutaneous AT from mice fed a ND or a HFD were collected, weighed and placed in KRBH buffer supplemented with 0.5 % BSA (2.5 ml/g of tissue) to be immediately processed. Human AT samples were collected from abdominal dermolipectomies in accordance with the recommendations of the ethics committee of Toulouse Hospital and revised (2000) declaration of Helsinki Principles (authorization DC-2017-2914). The mean age and body mass index (BMI) are as follow: lean (BMI = 22.6 ± 2.9 ; age = 48.6 ± 15.1) and obese (BMI = 32.6 ± 4.6 ; age = 47.3 ± 21.7). As for murine AT, human AT was weighed and placed in KRBH buffer supplemented with 0.5% BSA (2.5 mL/g of tissue) before rapid processing.

Human and murine AT were digested by collagenase $\Sigma 2$ (375 U/g tissue) for 30 min. After washing, adipocytes were isolated by flotation. For co-culture experiments, adipocytes (100 μ l) were embedded in a 3D fibrin matrix composed of fibrinogen (18 mg/ml; 100 μ l) and thrombin (25 U; 100 μ l) in a Falcon Cell Culture Insert. The culture dish was immediately placed at 37°C for 10 min to allow the gel to form before adding adipocyte culture medium (complete DMEM

supplemented with 50 nM insulin). Then, adipocytes were co-cultured, or not, for 5 days with melanoma cells seeded in the well below, or analyzed directly.

To condition medium, one million adipocytes per ml were suspended overnight in complete DMEM supplemented with 50 nM insulin.

Cell lines, culture and treatments

SKMEL28 melanoma cell line characteristics and culture conditions are described in suppM&M. For co-culture, 1.10^4 cells were cultured with adipocytes or treated with Ad-CM for 5 days. When indicated, cells were transfected with 50 nM siRNA in the presence of DharmaFECT1; 6 h post-transfection, fresh media, or Ad-CM, were added. Cells were used 48 h post-transfection.

EV isolation and cell treatment with EV

To prepare Ad-CM for EV isolation, complete DMEM was depleted of contaminating bovine vesicles by 100 000g overnight centrifugation. EV were purified from Ad-CM using centrifugations (3 000g for 30 min, then 90 min at 100 000g for the resulting supernatant). The pellets were suspended in EV-depleted DMEM for functional assays or washed in PBS for biochemical studies. To prepare EV-depleted CM, an additional 100 000g overnight centrifugation was performed. All centrifugations were carried out at 4°C. EV number were analyzed by Nanoparticle Tracking Analysis as previously described (Lazar et al., 2015).

COMPETING INTEREST

There are no conflicts of interest to declare.

ACKNOWLEDGEMENTS

We would like to thank S Milia and F Capilla for the immunohistochemistry experiments they performed at the Experimental Histopathology Facility of the INSERM/UPS CREFRE

Toulouse Purpan, France, the “Infrastructures en Biologie, Santé et Agronomie” (IBiSA) for Toulouse Réseau Imagerie-IPBS, and Anexplo-IPBS for animal facilities.

The study was supported by the ‘Ligue Régionale Midi-Pyrénées contre le Cancer’, ‘Fondation ARC pour la recherche sur le cancer’ and ‘Société Française de Dermatologie’. EC was a recipient of a PhD fellowship from ‘Ligue nationale contre le Cancer’, and EC and IL were recipients of PhD fellowships from ‘Fondation ARC pour la recherche sur le cancer’. LC benefits from a grant from ‘Fondation pour la Recherche Médicale’.

AUTHOR CONTRIBUTIONS

Conceptualization, EC, IL, CM, LL and LN, Methodology, LC, EC, IL, VD, NA and LN, Investigation, LC, EC, IL, SDau, MM and LN. Validation and Formal analysis, LC, EC, IL, DE, and LN, Writing, LC, EC, IL and L.N, Funding Acquisition, SDalle, LL, CM and LN, Resource, VD, LL and NA, Supervision, LN.

REFERENCES

- Ackermann J, Frutschi M, Kaloulis K, McKee T, Trumpp A and Beermann F. Metastasizing melanoma formation caused by expression of activated N-RasQ61K on an INK4a-deficient background. *Cancer research* 2005;6:4005-4011.
- Andarawewa KL, Motrescu ER, Chenard MP, Gansmuller A, Stoll I, Tomasetto C, et al. Stromelysin-3 is a potent negative regulator of adipogenesis participating to cancer cell-adipocyte interaction/crosstalk at the tumor invasive front. *Cancer Research* 2005;65:10862-10871.
- Boespflug A, Caramel J, Dalle S and Thomas L. Treatment of NRAS-mutated advanced or metastatic melanoma: rationale, current trials and evidence to date. *Therapeutic advances in medical oncology* 2017;9:481-492.
- Brandon EL, Gu JW, Cantwell L, He Z, Wallace G and Hall JE. Obesity promotes melanoma tumor growth: role of leptin. *Cancer biology & therapy* 2009;8:1871-1879.
- Cancer Genome Atlas Network. Genomic Classification of Cutaneous Melanoma. *Cell* 2015;161:1681-1696.
- Clement E, Lazar I, Muller C and Nieto L. Obesity and melanoma: could fat be fueling malignancy? *Pigment cell & melanoma research* 2017;30:294-306.
- Clement E, Lazar I, Attane C, Carrie L, Dauvillier S, Ducoux-Petit M, et al. Adipocyte extracellular vesicles carry enzymes and fatty acids that stimulate mitochondrial metabolism and remodeling in tumor cells. *The EMBO journal* 2020;39:e102525.
- Deleye Y, Cotte AK, Hannou SA, Hennuyer N, Bernard L, Derudas B, et al. CDKN2A/p16INK4a suppresses hepatic fatty acid oxidation through the AMPKalpha2-SIRT1-PPARalpha signaling pathway. *The Journal of biological chemistry* 2020;295:17310-17322.

Delmas V, Beermann F, Martinozzi S, Carreira S, Ackermann J, Kumasaka M, et al. Beta-catenin induces immortalization of melanocytes by suppressing p16INK4a expression and cooperates with N-Ras in melanoma development. *Genes Dev* 2007;21:2923-2935.

de Snoo FA and Hayward NK. Cutaneous melanoma susceptibility and progression genes. *Cancer letters* 2005;230:153-186.

Dirat B, Bochet L, Dabek M, Daviaud D, Dauvillier S, Majed B, et al. Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion. *Cancer Research* 2011;71:2455-2465.

Dhomen N, Reis-Filho JS, da Rocha Dias S, Hayward R, Savage K, Delmas V, et al. Oncogenic Braf induces melanocyte senescence and melanoma in mice. *Cancer cell* 2009;15:294-303.

Duong MN, Geneste A, Fallone F, Li X, Dumontet C and Muller C. The fat and the bad: Mature adipocytes, key actors in tumor progression and resistance. *Oncotarget* 2017;8:57622-57641.

Fang S, Wang Y, Dang Y, Gagel A, Ross MI, Gershenwald JE, et al. Association between Body Mass Index, C-Reactive Protein Levels, and Melanoma Patient Outcomes. *J Invest Dermatol* 2017;137:1792-1795.

Hayward NK, Wilmott JS, Waddell N, Johansson PA, Field MA, Nones K, et al. Whole-genome landscapes of major melanoma subtypes. *Nature* 2017;545:175-180.

Hodis E, Watson IR, Kryukov GV, Arold ST, Imielinski M, Theurillat JP, et al. A landscape of driver mutations in melanoma. *Cell* 2012;150:251-263.

Iyengar P, Espina V, Williams TW, Lin Y, Berry D, Jelicks LA, et al. Adipocyte-derived collagen VI affects early mammary tumor progression in vivo, demonstrating a critical interaction in the tumor/stroma microenvironment. *J Clin Invest* 2005;115:1163-1176.

Jakob JA, Bassett RL, Ng CS, Curry JL, Joseph RW, Alvarado GC, et al. NRAS mutation status is an independent prognostic factor in metastatic melanoma. *Cancer* 2012;118:4014-4023.

Koni M, Pinnaro V and Brizzi MF. The Wnt Signalling Pathway: A Tailored Target in Cancer. *International journal of molecular sciences* 2020;21:7697-7723.

Krimpenfort P, Quon KC, Mooi WJ, Loonstra A and Berns A. Loss of p16Ink4a confers susceptibility to metastatic melanoma in mice. *Nature* 2001;413:83-86.

Lafontan M. Historical perspectives in fat cell biology: the fat cell as a model for the investigation of hormonal and metabolic pathways. *American journal of physiology Cell physiology* 2012;302:C327-359.

Laurent V, Guerard A, Mazerolles C, Le Gonidec S, Toulet A, Nieto L, et al. Periprostatic adipocytes act as a driving force for prostate cancer progression in obesity. *Nature Communications* 2016;7:10230-10244.

Lazar I, Clement E, Ducoux-Petit M, Denat L, Soldan V, Dauvillier S, et al. Proteome characterization of melanoma exosomes reveals a specific signature for metastatic cell lines. *Pigment Cell Melanoma Res* 2015;28:464-475.

Lazar I, Clement E, Dauvillier S, Milhas D, Ducoux-Petit M, LeGonidec S, et al. Adipocyte Exosomes Promote Melanoma Aggressiveness through Fatty Acid Oxidation: A Novel Mechanism Linking Obesity and Cancer. *Cancer research*. 2016;76:4051-4057.

Le Lay S, Rome S, Loyer X and Nieto L. Adipocyte-derived extracellular vesicles in health and diseases: Nano-packages with vast biological properties. *FASEB Bioadv* 2021;3:407-419.

Longo M, Zatterale F, Naderi J, Parrillo L, Formisano P, Raciti GA, et al. Adipose Tissue Dysfunction as Determinant of Obesity-Associated Metabolic Complications. *International Journal of Molecular Sciences* 2019;20:2358-80.

Malvi P, Chaube B, Pandey V, Vijayakumar MV, Boreddy PR, Mohammad N, et al. Obesity induced rapid melanoma progression is reversed by orlistat treatment and dietary intervention: role of adipokines. *Molecular oncology* 2015;9:689-703.

Ming Z, Lim SY and Rizos H. Genetic Alterations in the INK4a/ARF Locus: Effects on Melanoma Development and Progression. *Biomolecules* 2020;10:1447-1465.

Nieman KM, Kenny HA, Penicka CV, Ladanyi A, Buell-Gutbrod R, Zillhardt MR, et al. Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nature Medicine* 2011;17:1498-1503.

Park J, Morley TS, Kim M, Clegg DJ and Scherer PE. Obesity and cancer-mechanisms underlying tumour progression and recurrence. *Nature reviews Endocrinology* 2014;10:455-465.

Ouchi N, Parker JL, Lugus JJ and Walsh K. Adipokines in inflammation and metabolic disease. *Nat Rev Immunol* 2011;11:85-97.

Petit V, Raymond J, Alberti C, Pouteaux M, Gallagher SJ, Nguyen MQ, et al. C57BL/6 congenic mouse NRAS(Q61K) melanoma cell lines are highly sensitive to the combination of Mek and Akt inhibitors in vitro and in vivo. *Pigment Cell Melanoma Res* 2019;32:829-841.

Reed JA, Loganzo F, Shea CR, Walker GJ, Flores JF, Glendening JM, et al. Loss of expression of the p16/cyclin-dependent kinase inhibitor 2 tumor suppressor gene in melanocytic lesions correlates with invasive stage of tumor progression. *Cancer research* 1995;55:2713-2718.

Rehman AG, Tyson M, Egger M, Heller RF and Zwahlen M. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. *Lancet* 2008;371:569-578.

Ruiz A, Puig S, Lynch M, Castel T and Estivill, X. Retention of the CDKN2A locus and low frequency of point mutations in primary and metastatic cutaneous malignant melanoma. *International journal of cancer* 1998;76:312-316.

Senni N, Savall M, Cabrerizo Granados D, Alves-Guerra MC, Sartor C, Lagoutte I, et al. beta-catenin-activated hepatocellular carcinomas are addicted to fatty acids. *Gut* 2019;68:322-334.

Sergentanis TN, Antoniadis AG, Gogas HJ, Antonopoulos CN, Adami HO, Ekblom A, et al. Obesity and risk of malignant melanoma: a meta-analysis of cohort and case-control studies. *Eur J Cancer* 2013;49:642-657.

Shain AH, Yeh I, Kovalyshyn I, Sriharan A, Talevich E, Gagnon A, et al. The Genetic Evolution of Melanoma from Precursor Lesions. *The New England journal of medicine* 2015;373:1926-1936.

Skowron F, Berard F, Balme B and Maucourt-Boulch D. Role of obesity on the thickness of primary cutaneous melanoma. *J Eur Acad Dermatol Venereol* 2015;29:262-269.

Sohier P, Legrand L, Aktary Z, Grill C, Delmas V, Bernex F, et al. A histopathological classification system of Tyr::NRAS(Q61K) murine melanocytic lesions: A reproducible simplified classification. *Pigment Cell Melanoma Res* 2018;831:423-431.

Stenehjem JS, Veierod MB, Nilsen LT, Ghiasvand R, Johnsen B, Grimsrud TK, et al. Anthropometric factors and Breslow thickness: prospective data on 2570 cases of cutaneous melanoma in the population-based Janus Cohort. *Br J Dermatol* 2018;179:632-641.

Talve L, Sauroja I, Collan Y, Punnonen K and Ekfors, T. Loss of expression of the p16INK4/CDKN2 gene in cutaneous malignant melanoma correlates with tumor cell proliferation and invasive stage. *International journal of cancer* 1997;74:255-259.

Zeng H, Jorapur A, Shain AH, Lang UE, Torres R, Zhang Y, et al. Bi-allelic Loss of CDKN2A Initiates Melanoma Invasion via BRN2 Activation. *Cancer cell* 2018;34:56-68 e59.

FIGURE LEGENDS

Figure 1: Obesity increases melanoma incidence and aggressiveness in GEMM models of melanoma

a) Mice bearing the Tyr::NRAS^{Q61K} transgene (NRAS) alone, or with heterozygous deletion of *Cdkn2a* (NRAS-p16), were fed normal (ND) or high fat (HFD) diets. Tyr prom: Tyrosinase promoter, Enh: enhancer, E: exon; Neo: neomycin.

b) Kaplan-Meier curves. Blue and brown stars compare ND and HFD mice in NRAS and NRAS-p16, respectively. ns compares NRAS HFD and NRAS-p16 ND.

c) Number of cutaneous melanomas in tumor-bearing mice.

d) Primary melanoma volume was measured weekly and the mean size increase was calculated.

e) Inguinal lymph node volume in melanoma bearing mice.

f) Presence of cutaneous melanoma and metastases per mouse.

Values are means \pm SD. Significance of differences: one-way ANOVA with post hoc Tukey's test, normality of samples: Kolmogorov-Smirnov test. For Kaplan-Meier curves [Log-rank (Mantel-Cox) test]. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: non-significant. $n=15$, except for NRAS ND in c-f ($n=6$).

Figure 2: Obesity decreases p16^{INK4A} in tumors

p16^{INK4A} immuno-staining in cutaneous melanoma isolated from mice fed a ND or a HFD.

a) Representative images, scale bar: 50 μ m.

b) Quantification ($n=10$, except for NRAS ND, $n=6$).

Values are means \pm SD. The significance of differences between the means was evaluated by one-way ANOVA, with the post hoc Tukey's test, normality of samples being determined using a Kolmogorov-Smirnov test. ** $p < 0.01$, *** $p < 0.001$, ns: non-significant.

Figure 3: Adipocytes decrease p16^{INK4A} expression in melanoma cells, a process amplified in obesity

SKMEL28 were cocultivated (CoC), or not (NC) with adipocytes or treated, or not (Ctrl) with Ad-CM from lean or obese (OB) individuals.

a-b: Cell motility (left) and WB analysis of p16^{INK4A} (middle) with quantifications (right).

Tubulin (TUB): loading control.

c) RT-qPCR analysis of p16^{INK4A}.

d) SKMEL28 cells were transfected, or not (NT), with 2 siRNA targeting p16^{INK4A} (sip16#1 & 2) or untargeted siRNA (siUT) followed by WB (top) or motility (bottom) analysis. Results are relative to untransfected cells of each group.

Values are means \pm SD (a-c: n=5, d: n=40). The significance of differences (evaluated by one-way ANOVA with post hoc Tukey's test, normality of samples being determined using a Kolmogorov-Smirnov test). *p < 0.05, **p < 0.01, *** p < 0.001, ns: non-significant.

Figure 4: Obesity increases adipocyte-dependent β -catenin content and activity in melanoma cells

a) SKMEL28 cells were cocultivated (CoC), or not (NC), with adipocytes or exposed, or not (Ctrl) to Ad-CM from lean or obese (OB) individuals. Then, β -catenin immunostaining was performed. Left, representative images. Right, quantification of the percentage of cells bearing nuclear β -catenin. Scale bar=50 μ m.

b-d: SKMEL28 cells were exposed, or not, to Ad-CM from lean or obese individuals.

b) TCF/LEF reporter assay. FOP (FOP-FLASH); TOP (TOP-FLASH).

c) β -catenin protein (β -cat), studied by WB. Tubulin (TUB): loading control. Left, representative experiments; Right, quantification.

d) RT-qPCR analysis of β -catenin.

Values are means \pm SD (n=5); statistically significant determined by one-way ANOVA (post hoc Tukey's test), normality of samples being determined using a Kolmogorov-Smirnov test. *p < 0.05, **p < 0.01, ***p < 0.001, ns: non-significant.

Figure 5: Ad-EV are responsible for the effect of Ad-CM on melanoma cells

a) Ad-CM from obese individuals was depleted, or not, of EV, EV depletion being verified by nanoparticle tracking analysis. CM (whole Ad-CM); SF (soluble factors) *i.e.* EV-depleted Ad-CM.

b-d: SKMEL28 cells were treated, or not (Ctrl) with Ad-CM from obese individuals, SF, or EV purified from Ad-CM.

b) β -catenin and p16^{INK4A} content in melanoma cells, studied by WB. Tubulin (TUB): loading control. Representative experiments (exp1 & 2, left) and quantifications (right) are shown.

c) β -catenin immunostaining. Scale bar: 50 μ m.

d) p16^{INK4A} expression analyzed by RT-qPCR.

Bars and error flags represent means \pm SD (n=5); statistically significant determined by one-way ANOVA (post hoc Tukey's test), normality of samples being determined using a Kolmogorov-Smirnov test. *p < 0.05, **p < 0.01, ***p < 0.001, ns: non-significant.

Figure 6: Ad-EV drive β -catenin-dependent p16^{INK4A} repression, a process amplified in obesity

a-b) EV from equivalent quantities of Ad-CM from lean or obese (OB) individuals were isolated.

a) Particle distribution (left) and concentration (right), analyzed by nanoparticle tracking.

b) EV protein analysis (FLOT1, ALIX and TSG101: EV markers).

c-d: SKMEL28 cells were treated, or not, with EV isolated from equal volumes of Ad-CM from lean or obese individuals.

c) β -catenin and p16^{INK4A} content in melanoma cells. Tubulin: loading control. Left, representative experiments. Right: quantifications.

d) β -catenin immunostaining. Left, representative images. Right, quantification of the percentage of cells bearing nuclear β -catenin. Scale bar: 50 μ m.

Values are means \pm SD (n=5); statistically significant determined by one-way ANOVA (post hoc Tukey's test), normality of samples being determined using a Kolmogorov-Smirnov test.

*p < 0.05, **p < 0.01, ***p < 0.001.

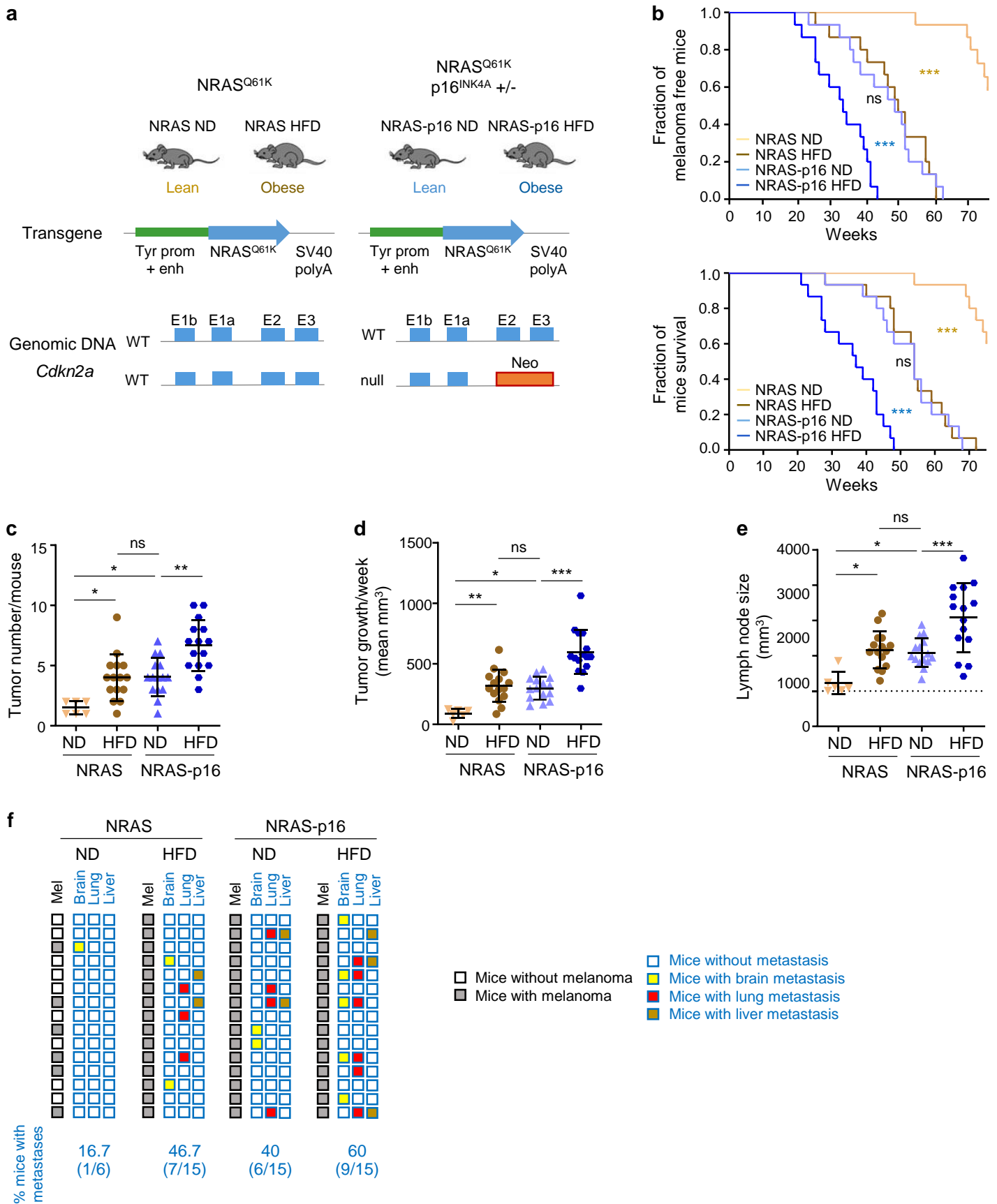


Figure 1

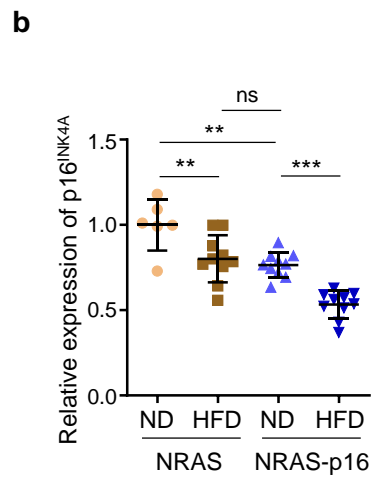
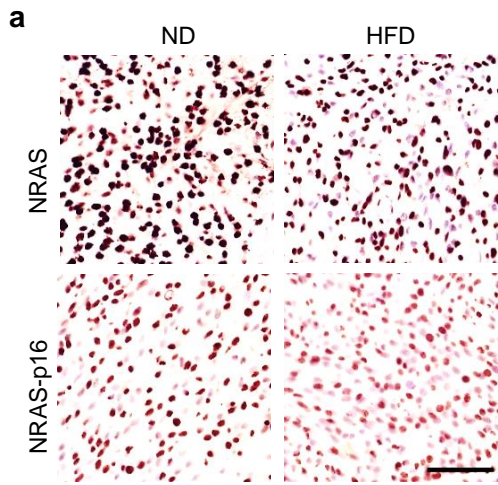


Figure 2

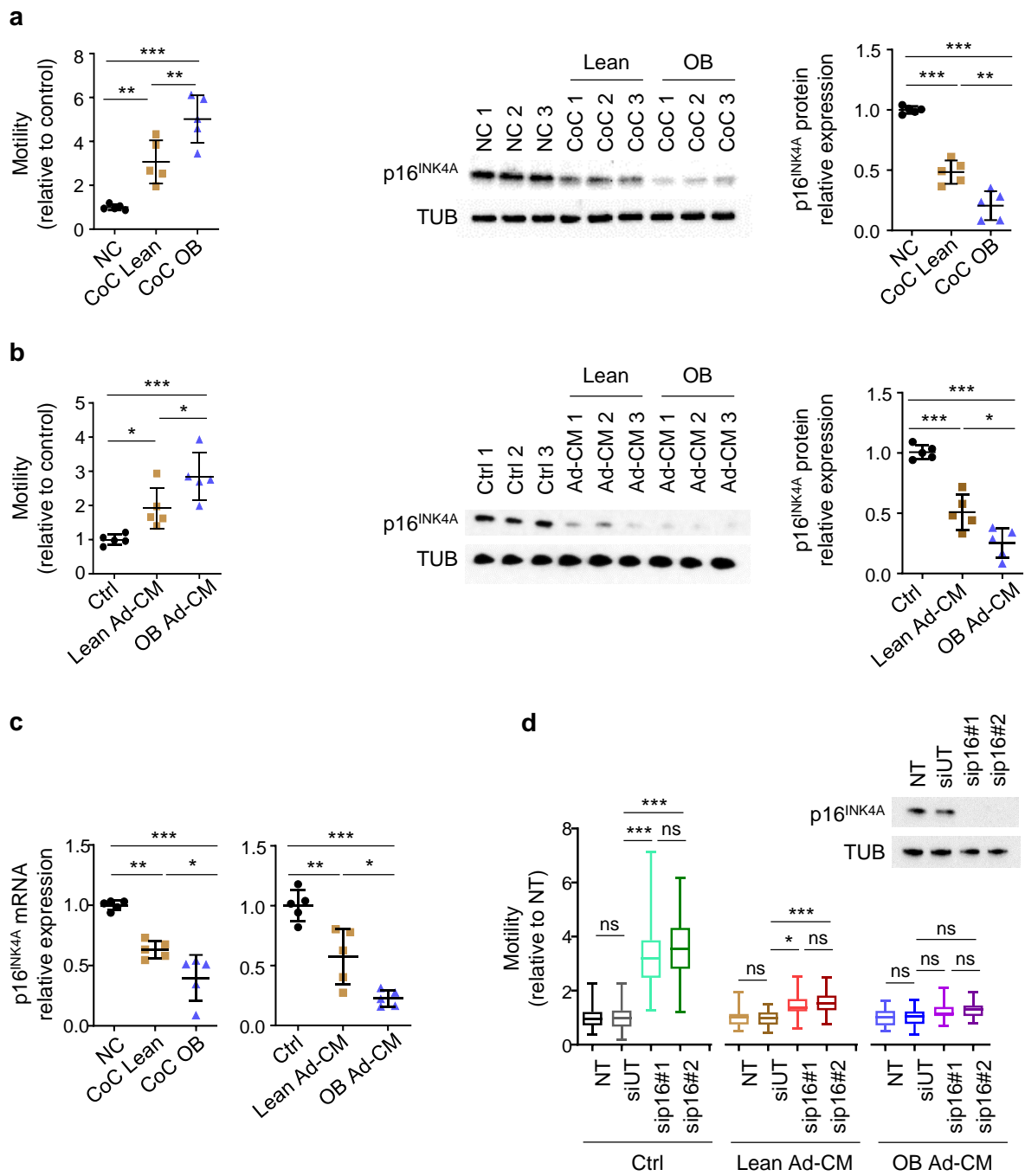


Figure 3

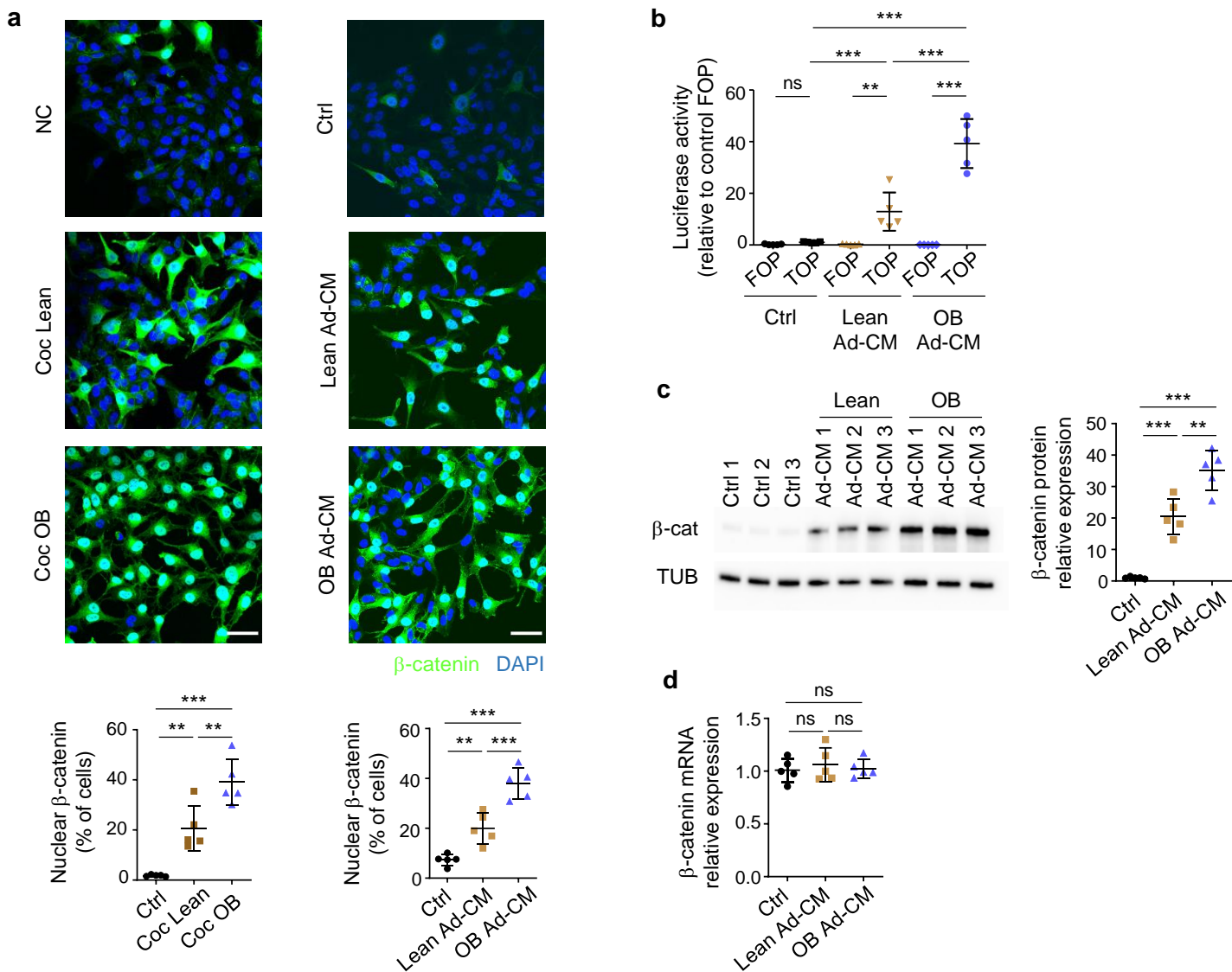


Figure 4

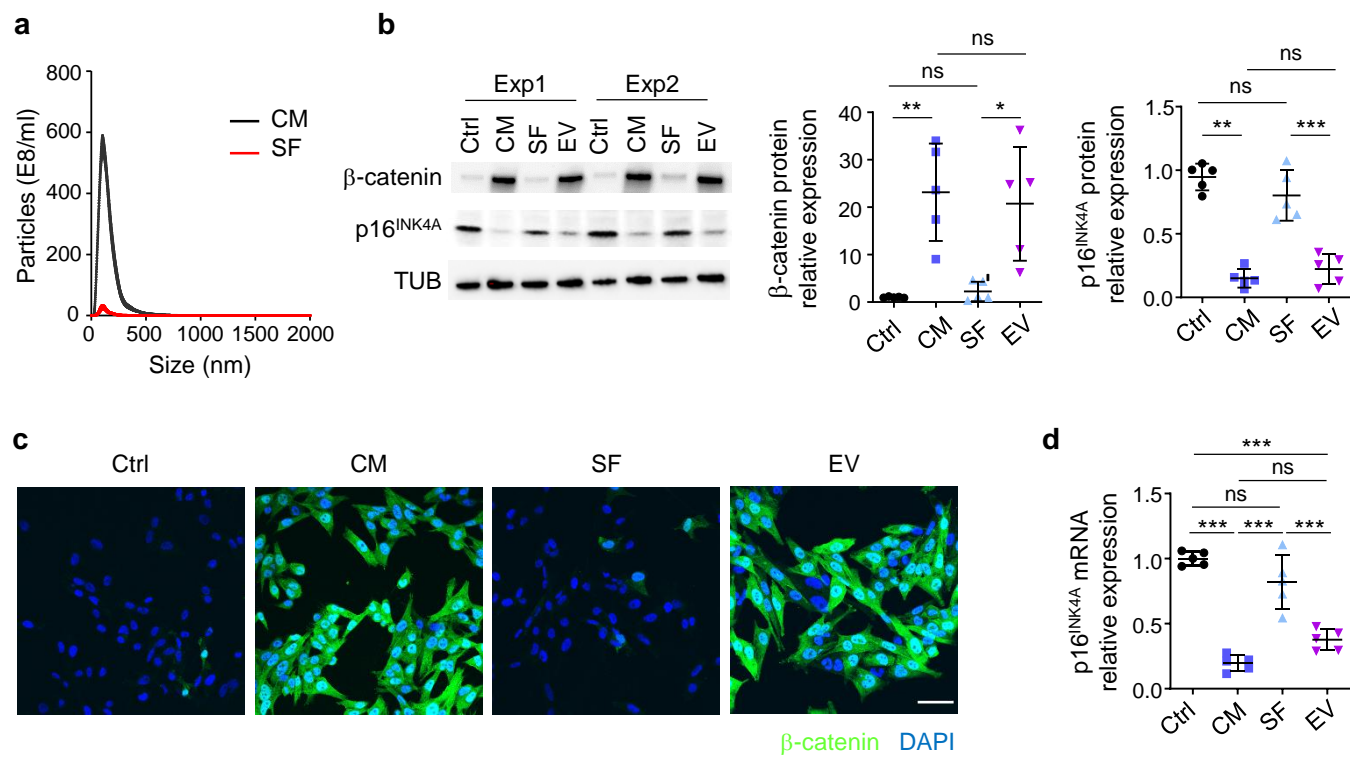


Figure 5

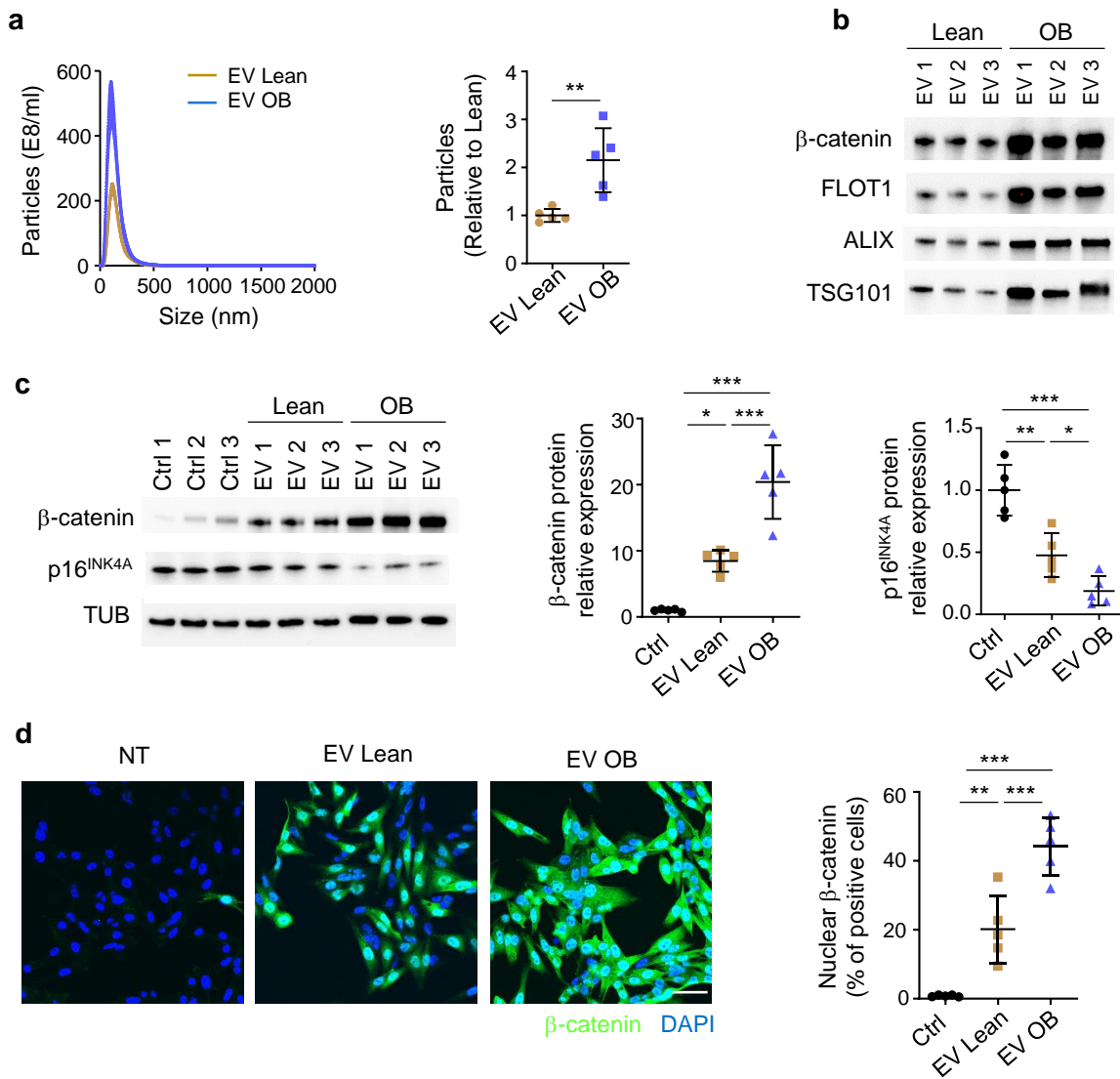


Figure 6