NADK Isoform 3 promotes oxidative stress resistance and melanoma metastasis

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Abstract

Metastasizing cancer cells encounter a multitude of stresses throughout the metastatic cascade. Oxidative stress is known to be a major barrier for metastatic colonization, such that metastasizing cancer cells must rewire their metabolic pathways to increase their antioxidant capacity. NADPH is essential for regeneration of cellular antioxidants and several NADPH-regenerating pathways have been shown to play a role in metastasis. However, we have found that metastatic melanoma cells have increased levels of both NADPH and $NADP^+$ suggesting increased *de novo* biosynthesis of $NADP^+$. *de novo* biosynthesis of $NADP^+$ occurs through a single enzymatic reaction catalyzed by NAD^+ kinase (NADK). Here we show that different NADK isoforms are differentially expressed in metastatic melanoma cells, with Isoform 3 being specifically upregulated in metastasis. We found that Isoform 3 is much more potent in expanding the NADP(H) pools, increasing oxidative stress resistance and promoting metastatic colonization compared to Isoform 1. Mechanistically, we have found that Isoform 3 is transcriptionally upregulated by oxidative stress and hypoxia through the action of NRF2 and HIF1a. Together, our work presents a previously uncharacterized role of NADK isoforms in oxidative stress resistance and metastasis and suggests that NADK Isoform 3 is a potential therapeutic target in metastatic disease.

Introduction

Oxidative stress limits metastasis of cancer cells[1]. Cancer cells, both in circulation and during metastatic colonization, experience high levels of oxidative stress and exhibit decreased levels of the cellular antioxidant, reduced glutathione (GSH)[2]. While most cancer cells do not survive the metastatic cascade, successful metastasizers are able to rewire their metabolism to upregulate antioxidant pathways and overcome oxidative stress by regenerating GSH from its oxidized form, GSSG. To reduce GSSG to GSH cells must utilize NADPH, in the process reducing it to $NADP^+$. It has been shown previously that metastasizing cell rely on different NADPH regeneration pathways including one carbon metabolism and pentose phosphate pathway, to increase their antioxidant capacity. However, how the process by which metastaszing cells replenish their pool of NADP(H) is unknown.

$NADP^+$ is generated *de novo* by NAD^+ kinase (NADK) by phosphorylating NAD^+[3,4] Due to a high cellular demand for reduction reactions, cells maintain a high NADPH/$NADP^+$ ratio to ensure that these reactions are favorable[5]. Since $NADP^+$ and NADPH are membrane impermeable cells have developed organelle-specific NADPH synthesis. In mammals, NADK is the cytoplasmic form of the enzyme, while NADK2 localizes to the mitochondria[6,7]. NADK contains a bifunctional $NADP^+$ phosphatase/NAD kinase domain, while NADK2 contains a diacylglycerol kinase catalytic domain[8]. Phylogenetic analysis indicates that NADK and NADK2 have developed independently during evolution[8]. Presence of other NADKs in organelles such as peroxisomes and endoplasmic

reticulum remains unknown. Recent studies demonstrated the importance of NADK in the antioxidant defense, while NADK2 has been mainly implicated in proline biosynthesis[9,10], which is why we have focused our study on the role of NADK in oxidative stress resistance in metastasis.

NADK exists in three isoforms. Isoform 1 and 2 are two longer isoforms that contain a long autoinhibitory N-terminal domain[11]. It has been previously shown that both Akt and PKC regulate the activity of Isoform 1 and 2 through phosphorylation of three terminal serine residues: Ser44, Ser46 and Ser48[11,12]. This phosphorylation relieves the autoinhibition of NADK and stimulates $NADP^+$ production. In contrast, Isoform 3 is a shorter isoform, generated through an alternative transcriptional start site with a unique N-terminal domain that does not have autoinhibitory activity or Akt/PKC phosphorylation sites, which is why Isoform 3 has much higher enzymatic activity than Isoform 1 at steady state[11]. However, the differential role of NADK isoforms in NADP generation is unknown.

Here we examined the role of NADK isoforms in oxidative stress resistance and metastasis to address how NADK activity contributes to *de novo* NADP(H) generation as an antioxidant defense mechanism in metastasizing melanoma cells. We specifically focused on Isoforms 1 and 3 as they were the only ones detectable in primary and metastasizing melanoma cells. We generated gain and loss of function melanoma cell lines of these NADK Isoforms which allowed us to address how these differential activities impacted melanoma cell survival under oxidative stress and during metastasis. We demonstrated that metastasizing melanoma cells increase NADP(H) biosynthesis and increase levels of Isoform 3 compared to the primary tumor. Overexpression of Isoform 3 decreased oxidative stress and protected melanoma cells against prooxidant treatment. Loss of Isoform 3 caused a greater increase in both cytoplasmic and mitochondrial oxidative stress levels than loss of isoform 1, and sensitized cells against prooxidant treatment. Finally, loss of either Isoform 1 or 3 had no effect on the growth of subcutaneous tumor but lead to a significant decrease in metastasis. Interestingly, overexpression of Isoform 1 or 3 had no significant effect on primary tumor growth either, but overexpression of truncated Isoform1 or Isoform 3 caused a much higher increase in metastatic burden in the organs of the mice compared to Isoform 1, while frequency of circulating tumor cells was lower in the blood of the mice with Isoform 3 overexpression compared to Isoform 1. We find that transcriptional upregulation of Isoform 3 is driven by hypoxia and specifically by transcription factors, NRF2 and HIF1a. Our findings suggest that metastasizing melanoma cells increase expression of NADK Isoform 3 to combat oxidative stress and increase their survival during the metastatic cascade. Our data indicate that increased levels of Isoform 3 promote melanoma cell extravasation and colonization of distant sites. Finally, our findings identify NADK isoform 3 as a potential therapeutic target for metastatic disease.

Results

Metastasis is limited by oxidative stress in several cancer types[1,2]. It has been previously shown that metastasizing melanoma cells rely on NADPH regenerating pathways such as one carbon metabolism and pentose phosphate pathway as part of their antioxidant defense system[2,13]. We have used a previously characterized patient-derived xenograft model of melanoma metastasis to quantify levels of NADPH and $NADP^+$ in subcutaneous tumors and metastatic nodules in two different patient derived melanoma xenografts with different driver mutations, M405 (NRAS Q61H) and M481 (BRAF V600E)[14]. We find that both NADPH and $NADP^+$ are elevated in metastatic nodules in different organs compared to the primary tumor in both PDX models (Fig

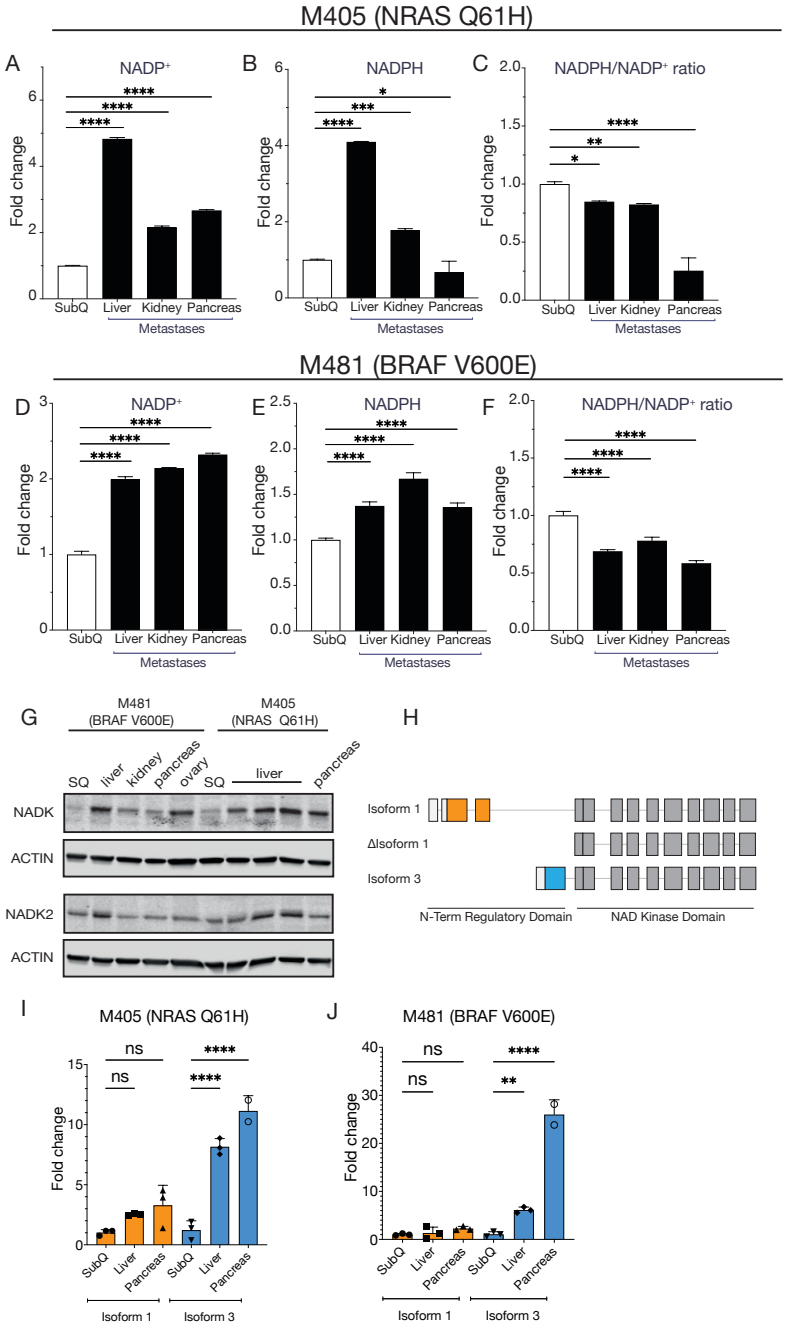

Figure 1: Metastatic nodules have increased synthesis of NADP+ and NADPH as well as upregulation of NAD+ Kinase

(A) Levels of NADP+ and (B) NADPH measured by luminescence and (C) ratio of NADPH/NADP+ in subcutaneous melanoma tumors compared to metastatic nodules from different organs in PDX tumor M405. One-way ANOVA, p-value *<0.05, **<0.005, ***<0.0005, ****<0.00005

(D) Levels of NADP+ and (E) NADPH measured by luminescence and (F) ratio of NADPH/NADP+ in subcutaneous melanoma tumors compared to metastatic nodules from different organs in PDX tumor M481. One-way ANOVA, p-value *<0.05, **<0.005, ***<0.0005, ****<0.00005

(G) Expression levels of NAD+ Kinase and NAD+ Kinase 2 in subcutaneous melanoma tumors compared to metastatic nodules in PDX melanoma tumors M405 and M481 measured by western blot.

(H) Schematic of NAD+ Kinase Isoforms 1, truncated Isoform 1 and Isoform 3.

Expression levels of Isoform 1 and 3 measured by RT-qPCR in PDX tumors M405 (I) and M481 (J) in subcutaneous tumors compared to metastatic nodules. Unpaired t-test, p-value **<0.005, ****<0.00005

1 A, B and Fig 1 D, E). The ratio of NADPH/NADP+ is an indicator of oxidative stress levels, where a low NADPH/NADP+ ratio indicates high levels of oxidative stress in the cells. We have found that the ratio of NADPH/NADP+, was decreased in metastatic nodules compared to the subcutaneous tumor in both PDX models, indicating higher levels of oxidative stress (Fig 1 C and F). Overall, these data suggest that while metastases are under increased levels of oxidative stress, they increase regeneration of NADPH from NADP+ as well as *de novo* biosynthesis of NADP+ to sustain increased antioxidant capacity. We have also found increased levels of both NAD+ and NADH in metastatic nodules compared to subcutaneous tumors across two different PDX models of melanoma metastasis (Supp Fig 1 A, B, D and E), consistent with increased *de novo* NADP+ biosynthesis, however the NAD+/NADH ratio remained unchanged (Supp Fig 1 C and F).

Since the main mechanism of *de novo* NADP+ biosynthesis is through NADK activity, we tested expression levels of both cytoplasmic NADK and mitochondrial NADK2 in metastatic nodules and subcutaneous tumors in two different PDX models of melanoma metastasis (Fig 1G). Metastatic nodules showed a significant increase in cytoplasmic NADK levels and no increase in mitochondrial NADK2 levels, indicating increased NADP+ biosynthesis in the cytoplasm during metastasis.

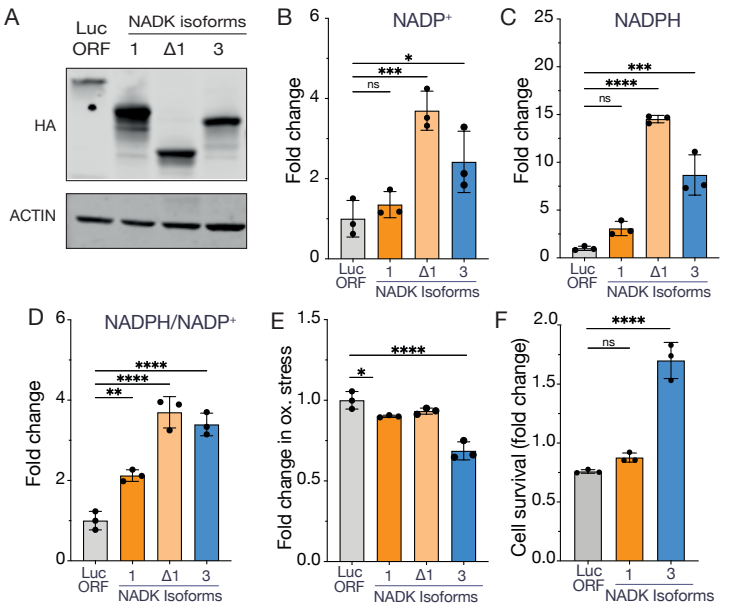

Figure 2: NADK Isoform 3 protects cells against oxidative stress
(A) Overexpression of NADK isoforms in A375 melanoma cells by western blot.
(B) Levels of $NADP^+$ and (C) NADPH, and (D) ratio of NADPH/$NADP^+$ in A375 melanoma cell lines overexpressing NADK isoforms. One-way ANOVA, p-value *<0.05, **<0.005, ***<0.0005, ****<0.00005
(E) Oxidative stress levels measured by flow cytometry using CellROX Green dye in A375 melanoma cells overexpressing NADK isoforms. One-way ANOVA, p-value *<0.05, ****<0.00005
(F) Survival of A375 melanoma cells under oxidative stress from pyocyanin treatment (10uM) for 24 hr expressing NADK isoforms measured by Cell Titre Glo luminescence. One-way ANOVA, p-value ****<0.00005

Since NADK has three different isoforms, we tested the expression level of the different isoforms by RT-qPCR in metastatic nodules compared to the primary tumor in the different PDX models of melanoma metastasis. Isoforms 1 and 2 contain a long autoinhibitory N-terminal domain, while Isoform 3 has a different transcriptional start site and has a unique N-terminal domain (Fig 1H). Due to the difference in the N-terminus domains, Isoform 3 has been previously shown to have a much higher activity than Isoform 1 and 3. Truncated Isoform 1(ΔIsoform 1) (Fig 1H) has also been shown to have a similar activity level as Isoform 3 *in vitro*. This suggests that the autoinhibitory function of the Isoform 1 N-terminus drives the differential activity of Isoform 1 and 3. Isoform 2 was not detectable in any of the tissues (Data not shown) so we focused on the role of Isoform 1 and 3 in metastasis. We found that Isoform 3 was greatly upregulated in metastatic nodules compared to Isoform 1 suggesting that the observed increase in $NADP^+$ biosynthesis in metastasis maybe due to increased activity of Isoform 3 (Fig 1I and J).

To compare the role of Isoform 1 and 3 in oxidative stress resistance, we overexpressed HA-tagged Isoform 1, truncated Isoform 1 (ΔIso1), which is missing its regulatory N-terminal domain mimicking Isoform 3, and Isoform 3 in A375 melanoma cells (Fig 1H and 2A). Firefly luciferase was used as a control. While overexpression of wild-type Isoform 1 caused a modest increase in NADPH/$NADP^+$ ratio, both truncated Isoform 1 and Isoform 3 caused a significant increase in $NADP^+$, NADPH and NADPH/$NADP^+$ ratio (Fig 2B-D). This is consistent with previously published reports that showed that truncated Isoform 1 and Isoform 3 have similar activity *in vitro*, and that the autoinhibitory N-terminus domain of Isoform 1 keeps its activity low unless phosphorylated by upstream kinases such as Akt and PKC. We also measured levels of NAD^+ and NADH in Isoform-overexpressing cells and found that both NAD^+ and NADH were decreased in ΔIso1 and Isoform 3 overexpressing cells but not in full-length Isoform 1 overexpressing cells, consistent with increased $NADP^+$ biosynthesis (Supp Fig 2A and B). However the ratio of NAD/NADH did not change in any of the cell lines suggesting there is no major changes in NAD/NADH

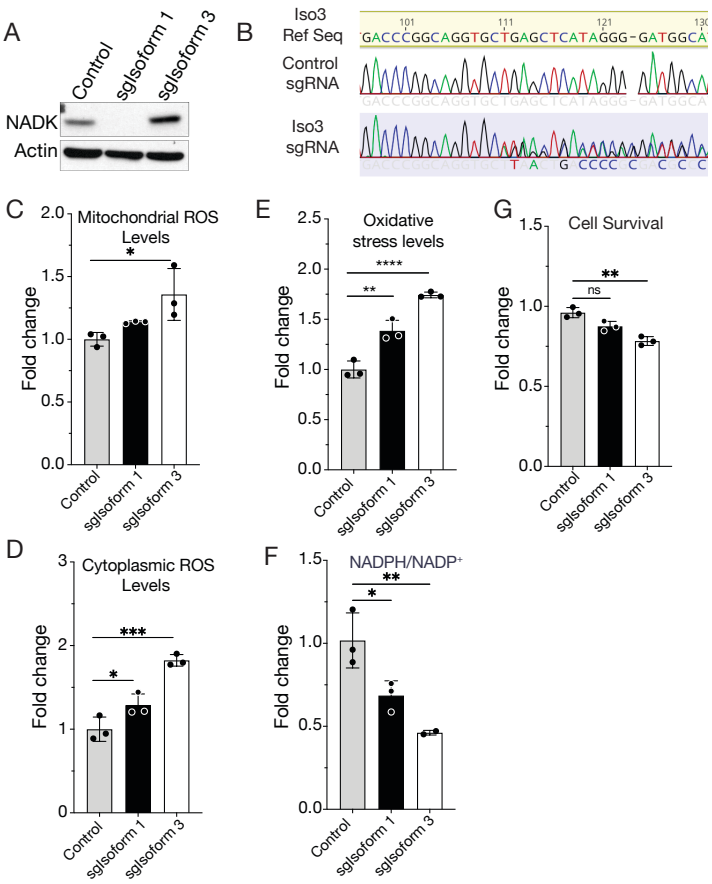

Figure 3: Loss of NADK Isoform 3 increases oxidative stress
(A) Levels of NADK Isoform 1 in A375 melanoma cells with Isoform 1 or Isoform 3 CRISPR/Cas9 deletions measured by western blot.
(B) Sequencing of genomic DNA from either Control or Isoform 3 CRISPR/Cas9 deleted A375 melanoma cells.
(C) Levels of mitochondrial ROS levels measured by flow cytometry using CellROX Green dye in A375 melanoma cells with either Isoform 1 or Isoform 3 CRISPR/Cas9 deletions. One-way ANOVA, p-value *<0.05
(D) Levels of cytoplasmic ROS levels measured by flow cytometry using CellROX DeepRed dye in A375 melanoma cells with either Isoform 1 or Isoform 3 CRISPR/Cas9 deletions. One-way ANOVA, p-value **<0.005, ****<0.00005
(E) Oxidative stress levels in A375 melanoma cells with either Isoform 1 or Isoform 3 deletion treated with Pyocyanin (10 uM) for 24 hr measured by flow cytometry using CellROX Green dye. One-way ANOVA, p-value **<0.005,
(F) Ratio of NADPH/NADP$^+$ in A375 melanoma cells with Isoform 1 or Isoform 3 CRISPR/Cas9 deletions measured by luminescence. One-way ANOVA, p-value *<0.05, **<0.005
(G) Survival of A375 melanoma cells with either Isoform 1 or Isoform 3 deletion under oxidative stress after treatment with prooxidant H_2O_2 (20uM) for 24 hr measured by Cell-Titre Glo luminescence. One-way ANOVA, p-value **<0.005

homeostasis (Supp Fig 2C). Since we observed an increased NADPH/NADP$^+$ ratio, we wanted to assess the effect of overexpression of different NADK isoforms on levels of cellular oxidative stress using CellROX Green ROS reactive flow cytometry dye. Intriguingly, only cells overexpressing Isoform 3 had significantly lower oxidative stress levels compared to cells overexpressing Isoform 1 or truncated Isoform 1, as detected by flow cytometry (Fig 2E). This suggests that although truncated Isoform 1 and Isoform 3 have similar activity, they have different effect on cellular oxidative stress levels presumably due to the unique N-terminus of Isoform 3. Consistent with this data, when A375 melanoma cells were treated with prooxidant pyocyanin, cells overexpressing Isoform 3 survived significantly better than control or Isoform 1- overexpressing cells (Fig 2F). This also suggests that the autoinhibitory domain of NADK isoform 1 limits cells ability to respond to oxidative stress.

To establish how the loss of individual NADK isoforms affects melanoma cells we used CRISPR/Cas9 technology to generate isoform-specific knockout A375 cell lines. Isoform 1 knockout cells were validated by western blotting (Fig 3A), however due to lack of Isoform 3-specific antibodies, genomic DNA sequencing was used to validate Isoform 3 knockouts (Fig 3B). Interestingly, we saw an upregulation of Isoform 1 levels in the Isoform 3 deleted cells suggesting that melanoma cells try to compensate for loss of Isoform 3 with Isoform 1 upregulation. We found that knockout of Isoform 3 specifically lead to an increase in mitochondrial oxidative stress levels while loss of Isoform 1 had no effect (Fig 3C). Knockout of Isoform 3 also caused a greater increase in cytoplasmic oxidative stress levels than Isoform 1 knockout (Fig 3D).

Oxidative stress induced by prooxidant pyocanin was much higher in cells lacking Isoform 3 than Isoform 1 as measured by flow cytometry using CellROX Green dye (Fig 3E). Consistent with these findings, NADPH/ $NADP^+$ ratio was much lower in cells lacking Isoform 3 than Isoform 1 (Fig 3F). Consistent with decreased NADP+ biosynthesis, we found increased levels of both NAD and NADH in cells lacking either Isoform 1 or Isoform 3 (Supp Fig 3 A and B). However, we did not find any changes in the NAD/NADH ratio (Supp Fig 3C). We tested viability of melanoma cells under treatment with prooxidant H_2O_2 and found that cells lacking Isoform 3 showed decreased cell survival while cells lacking Isoform 1 were not affected. Taken together, these data show that loss of Isoform 3 specifically elevates cellular oxidative stress through decreased NADPH/ $NADP^+$ ratio and sensitizes melanoma cells to oxidative stress. It also indicates that melanoma cells specifically depend on Isoform 3 for oxidative stress resistance and that Isoform 3 activity has a greater contribution to the cellular antioxidant defense than Isoform 1.

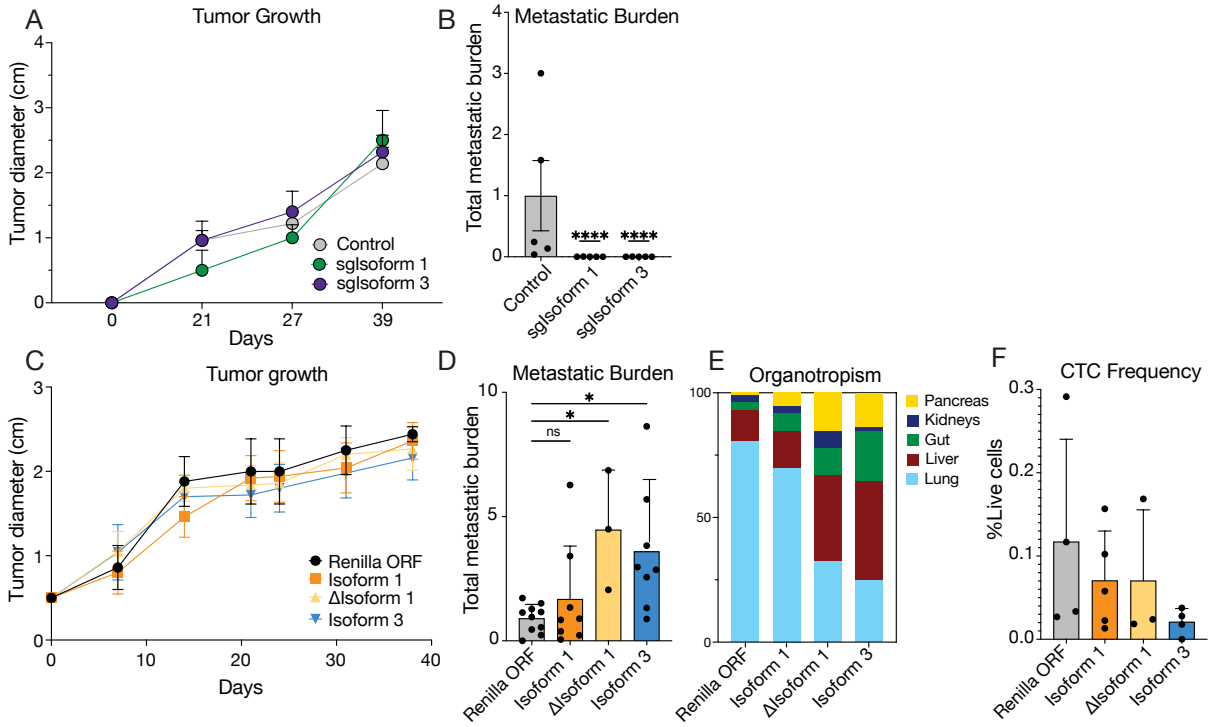

Figure 4: NADK Isoform 3 is necessary and sufficient for metastasis in vivo
(A) Subcutaneous tumor growth rate of A375 melanoma cells with either Isoform 1 or Isoform 3 deletion in NSG mice.
(B) Metastatic burden in the organs of NSG mice xenografted with A375 melanoma cells with either Isoform 1 or Isoform 3 deletion. One-sample t-test, p-value ****<0.00005
(C) Subcutaneous tumor growth of A375 melanoma cells overexpressing different NADK isoforms in NSG mice.
(D) Metastatic burden in the organs of NSG mice xenografted with A375 melanoma cells overexpressing different NADK isoforms. One-way ANOVA, p-value *<0.05
(E) Metastatic organotropism in the organs of NSG mice xenografted with A375 melanoma cells overexpressing different NADK isoforms.
(F) Circulating tumor cell frequency in the blood of NSG mice xenografted with A375 melanoma cells overexpressing different NADK isoforms.

Since we observed differential expression of NADK isoforms in metastasis, we wanted to functionally test the role of NADK isoforms in enabling survival of metastasizing melanoma cells during organ colonization *in vivo*. We labelled A375 melanoma cells using a dual reporter

expressing luciferase for bioluminescence imaging and a fluorescence protein dsRed for flow cytometry selection of melanoma cells. We transplanted labelled A375 melanoma cells lacking either Isoform 1 or Isoform 3 subcutaneously into immunocompromised mice and evaluated metastatic burden in the organs of the mice using bioluminescence once the subcutaneous tumor reached the end point after 6 weeks. We found that subcutaneous tumor growth was not affected by either Isoform 1 or Isoform 3 loss (Fig 4A). However, metastasis was greatly reduced by loss of either Isoform 1 and Isoform 3 suggesting that both isoforms are involved in metastatic colonization (Fig 4B).

To test whether NADK isoforms can differentially drive metastasis *in vivo*, we overexpressed different NADK isoforms in labelled A375 melanoma cells and transplanted them subcutaneously. None of the isoforms had a significant impact on the growth of the subcutaneous tumor (Fig 4C). Overexpression of wild type Isoform 1 had no effect on the amount of metastatic burden in the organs of the mice, however, overexpression of either truncated Isoform 1 or Isoform 3 significantly increased the metastatic burden in the organs of the mice (Fig 4D). This suggests that overall increased *de novo* biosynthesis of $NADP^+$ is a metastatic driver. When we analyzed the metastatic organotropism of melanoma cells overexpressing different isoforms, we found that both truncated Isoform 1 and Isoform 3 showed significantly less metastasis to the lung and more metastasis to other organs such as the liver, pancreas and

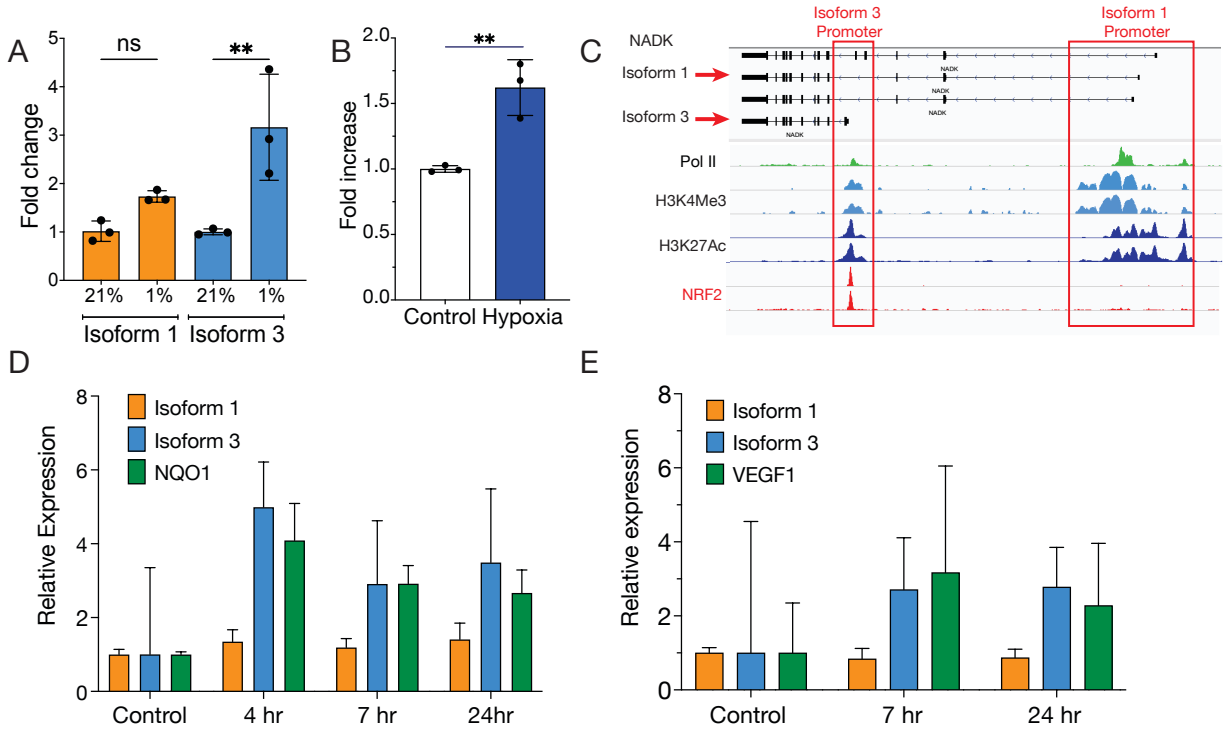

Figure 5: NADK Isoform 3 is regulated by hypoxia through NRF2 and HIF1a

(A) Expression of Isoform 1 and Isoform 3 in A375 melanoma cells grown in normoxic control (20% O_2) conditions compared to hypoxic (1% O_2) conditions measured by qPCR. One-sample t-test p-value **<0.005

(B) Levels of oxidative stress as measured by CellROX Green dye by flow cytometry in A375 melanoma cells in hypoxic (1% O_2) compared to normoxic control conditions (20% O_2). One-sample t-test p-value **<0.005

(C) ChIP-Seq analysis of markers of active transcription such as Pol II, H3K4Me3 and H3K27Ac, used to identify promoters of Isoform 1 and Isoform 3, as well as NRF2 binding to Isoform 3 promoter but not Isoform 1 in HeLa-S3 cells.

(D) Expression levels of Isoform 1, Isoform 3 and NQO1 after NRF2 activation with 10uM Ki696 treatment in A375 melanoma cells.

(E) Expression levels of Isoform 1, Isoform 3 and VEGF after HIF1 induction with 150uM CoCl2 in A375 melanoma cells.

the gut, compared to Isoform 1-overexpressing cells and control cells (Fig 4E). This indicates that increased *de novo* $NADP^+$ biosynthesis by truncated Isoform 1 or Isoform 3 drives broad metastatic colonization to different organs. This data also suggests that cells with low $NADP^+$ biosynthesis are lodged in the lung and are unable to metastasize more widely to other sites. Intriguingly, only overexpression of Isoform 3 lead to a decreased frequency of circulating tumor cells in the blood of the mice compared to control, wild type Isoform 1, or truncated Isoform 1 overexpressing cells (Fig 4F). This suggests that increased overexpression of Isoform 3 specifically promotes metastatic colonization through increased extravasation of melanoma cells from the blood into distant organs and points towards a unique role of Isoform 3 in the metastatic cascade.

Given the differential organotropism patterns between Isoform 1 and Isoform 3 overexpressing cells, we speculated that Isoform 3 must provide an advantage to cells during extravasation and metastatic colonization. One of the stresses that cells experience during early stages of seeding and colonization is hypoxia due to lack of vasculature and limited oxygen diffusion. We tested the effect of hypoxia (1% O_2) on the expression levels of NADK Isoforms and found that Isoform 3 was specifically upregulated in hypoxic conditions while Isoform 1 remained unaffected (Fig 5A). Given the role of NADK in oxidative stress, we tested oxidative stress levels in hypoxia and found that they were increased in the melanoma cells (Fig 5B). This suggested that NADK Isoform 3 maybe regulated by both oxidative stress and hypoxia. Since NRF2 is the major transcriptional regulator of the antioxidant response we analyzed existing ChIP data from the ENCODE database and found that NRF2 was specifically found to bind the putative promoter of Isoform 3 but not Isoform 1 in Hela-S3 cells (Fig 5C) and A549 cells (Data not shown). To test this hypothesis functionally, we used NRF2 activator Ki696 to test its effect on Isoform 3 induction. We found that NRF2 activation lead to an increase in Isoform 3 levels comparable to known NRF2 targets such as NQO1. Since we observed an increase in Isoform 3 levels in hypoxia, we also speculated that HIF1a might also play a role in the transcriptional upregulation of Isoform 3. We induced HIF1a activation using $CoCl_2$ and saw an acute upregulation of Isoform 3, comparable to its known target VEGF. This data suggests that Isoform 3 is upregulated in hypoxic conditions and in response to oxidative stress, and that it is specifically induced through the action of both NRF2 and HIF1a.

Discussion

Metastasizing cancer cells have increased plasticity and are able to rewire their metabolism to increase survival during metastatic colonization. Increased antioxidant capacity enables metastasis of cancer cells, by increasing regeneration of GSH from GSSG through use of NADPH and its consequent conversion to $NADP^+$. While there are numerous metabolic pathways that can recycle NADPH from $NADP^+$, to drive the cellular antioxidant cycle, such as pentose phosphate pathway, malic enzyme and one carbon metabolism, there are limited ways that the $NADP^+$ pool can be replenished *de novo*. NAD kinase (NADK) is the main route of generating $NADP^+$ from NAD^+. Since NADPH is used for reduction reactions, cells maintain a high NADPH/ $NADP^+$ ratio and therefore convert most of $NADP^+$ into NADPH. Increased activity of NADK would contribute to increased antioxidant capacity of cancer cells, through *de novo* synthesis of $NADP^+$ and therefore generation of NADPH for GSH regeneration.

NADK kinase has been shown to exist in several isoforms. Isoforms 1 and 2 have a common long-term autoinhibitory N-terminus domain, whose inhibition is alleviated upon post-translational phosphorylation. One of the major regulators of NADK activity has been shown to

be Akt signaling as well as PKC signaling. Both kinases have been shown to phosphorylate specific sites in the autoinhibitory N-terminus, which means that increased signaling through the Akt and PKC pathways leads to increased NADK activity and increased $NADP^+$ production. In contrast, Isoform 3 of NADK has a unique short N-terminal domain, generated by an alternative transcriptional start site, without autoinhibitory properties or targeting by upstream kinases. This also means that Isoform 3 has a much higher activity than the other Isoforms at baseline. Akt signaling through Akt1 has been shown to decrease during epithelial-mesenchymal transition[15,16]. In fact, genetic depletion of Akt1 has been shown to promote an invasive phenotype in breast cancer[15]. This suggests that there may not be sufficient Akt signaling to alleviate the autoinhibition of NADK Isoform 1 during metastasis, however metastasizing cells are still highly dependent on the NADP(H) pool for antioxidant defenses. Our findings suggest that Isoform 3 due to its high activity, which is independent of Akt signaling, is a potentially novel mechanism to increase the NADP(H) pool and increase oxidative stress resistance.

Our data suggest that both Isoform 1 and 3 are required for metastatic colonization, while loss of either isoform does not have an impact on the primary tumor growth. This may indicate that the demand for NADPH is much higher in metastasizing melanoma cells compared to proliferating cells in the primary tumor. This also suggests that even a minor decrease in $NADP^+$ levels has a strong effect on the ability of melanoma cells to colonize distant sites.

Interestingly, both truncated Isoform 1 and Isoform 3 overexpression lead to an increase in the metastatic burden in the organs of the mice, while Isoform 1 overexpression had no effect. Since it has been previously shown that truncated Isoform 1 and Isoform 3 have similar activities *in vitro*, and they both increase metastatic colonization, this data suggests that increased *de novo* $NADP^+$ generation is a major metastatic driver. Interestingly, when we analyzed metastatic organotropism we saw similar patterns with increased metastasis to the liver, gut and pancreas and decreased metastasis to the lung for truncated Isoform 1 and Isoform 3. On the other hand, control cells and full-length Isoform 1 overexpressing cells showed increased metastasis to the lung and limited metastasis to other organs. This suggests that during metastasis many cells get lodged in the lung and tend to metastasize there, however increased $NADP^+$ biosynthesis enables cells to metastasized more widely. It is possible that after dissemination, cells with increased $NADP^+$ biosynthesis are ablet survive better across many different organs, and therefore cause more widespread metastasis. Surprisingly, overexpression of Isoform 3 uniquely lead to a decreased frequency of circulating tumor cells in the blood, compared to Isoform 1 or truncated Isoform 1, suggesting that increased levels of Isoform 3 drive extravasation of melanoma cells from the blood into distant sites. This is consistent with the unique ability of Isoform 3 to lower oxidative stress in melanoma cells compared to Isoform 1 or truncated Isoform 1, suggesting that this function plays a unique role in metastatic cell extravasation. More broadly, this indicates that there are different mechanisms of oxidative stress resistance that are required for melanoma cells at different steps of the metastatic cascade.

Metastasizing cancer cells experience many different stresses during the metastatic cascade. It is likely that these stress cues induce specific stress resistance mechanisms. Our work specifically identified NRF2, a master regulator of the antioxidant response as well as HIF1a, a master regulator of the hypoxia response as transcription factors responsible for driving Isoform 3 expression. This suggests that upregulation of Isoform 3 maybe part of a broader network of oxidative stress and hypoxia adaptations that occur during metastatic colonization.

In conclusion, our work has discovered an antioxidant mechanism through differential transcriptional induction of different NADK isoforms. Isoform 3 is specifically upregulated in metastatic nodules compared to the primary tumor and provides antioxidant protection to melanoma cells increasing extravasation and organ colonization by metastasizing melanoma cells, driven by coordinated action of NRF2 and HIF1a. Taken together, our data highlight NADK Isoform 3 as a potential novel mechanism that drives metastatic colonization that could be exploited for therapeutic inhibition.

Methods

Plasmid Construction

miR-E shRNA knockdown constructs were generated by the splashRNA program and ordered from Invitrogen as oligos. Hairpins were ligated (T4 Ligase, NEB) into SiREP lentiviral plasmid behind an SFFV promoter and iRFP fluorescent protein using XhoI and EcoRI sites. For overexpression constructs, the template plasmids for NADK isoforms were kindly provided by Gerta Hoxhaj and the coding sequences of NADK isoforms 1 and 3 for the rescue experiments were ordered as gene fragments (IDT) with an N-terminus Kozack sequence and 3xFLAG. Constructs were ligated (In-Fusion HD, Takara or T4 Ligase, NEB) into pLenti vector behind an EFS promoter using BamHI and NsiI sites. A cleavable P2A site links the protein expression to a fluorescent protein tag. CRISPR knockout sgRNA sequences were generated using Geneious cloning software and ordered as single stranded oligos from IDT. Vector backbones were kindly provided by Lukas Dow, and all plasmids were sequence verified.

Lentiviral transduction and generation of stable cell lines

For virus production, 0.9µg of plasmid was combined with 1µg of packaging plasmids (0.4µg pMD2G and 0.6µg psPAX2) and transfected into HEK 293T cells using Polyjet (SignaGen) according to manufacturer's instructions. Fresh media was added the following day and viral supernatants were collected 72hr after transfection and filtered through a 0.45µM filter. Approximately 1 million cells were infected with viral supernatant and 10µg/mL polybrene (Sigma-Aldrich). Cells then underwent antibiotic selection with puromycin, hygromycin or blasticidin (Inviogen) depending on the antibiotic resistance of each plasmid. For CRISPR knockout, cells stably-expressing Cas9 were infected with the gRNA construct. To generate single clones, cells were diluted and plated into a 10cm dish. Glass rings were used to isolate single cells and grow colonies. All single clones were verified by western blot analysis and Synthego genomic DNA sequencing.

Real- Time PCR Quantification of mRNA

mRNA was extracted using a Direct-zol RNA Purification Kit (Zymo Research). Reverse transcription was performed using iScript cDNA synthesis kit (Rio-Rad) as described by manufacturer. cDNA was then diluted and used for qPCR analysis with SYBR Green PCR master mix (Thermo Fisher). IDT online primer design tool was used to generate qPCR primers and UCSC In-Silico PCR database was then used to verify human-specificity of primers. qPCR analysis was performed on an Applied Biosystems QuantStudio 6 Real-Time PCR System. All targets were normalized to Actin in a standard ΔΔCt analysis.

Analysis of cell survival under prooxidant treatment

Cells were plated at 10,000 cells/well in a white TC-treated 96 well plate (Corning). After 24 hours, cells were then treated with prooxidant pyocyanin (Cayman), H_2O_2 (Sigma-Aldrich). After 24 hours of treatment, CellTiter-Glo 2.0 (Promega) was used according to manufacturers instructions to quantify cell viability.

Lentiviral transduction of human melanoma cells

A lentiviral construct with luciferase and dsRed (luc P2A dsRed) was used to label patient-derived melanoma cells as described in Piskounova et al. Nature (2015). Virus was produced as described above. For lentiviral transduction, 500,000 freshly dissociated melanoma cells were infected with viral supernatant and supplemented with 10 µg/mL polybrene (Sigma). The following day, the media was replaced and 48hrs post-infection cells were either injected subcutaneously into mice as bulk tumors or FACS sorted for positive infection.

Flow cytometry of melanoma cells

All melanoma cells in this study stably express dsRed and luciferase so that melanoma cells could be distinguished by flow cytometry or bioluminescent imaging. When preparing cells for sorting by flow cytometry, cells were stained with antibodies against mouse CD45 (30-F11-VioletFluor, Tonbo), mouse CD31 (390-VioletFluor, eBiosciences), Ter119 (Ter-119-VioletFluor, Tonbo) and human HLA-A, -B, -C (BD Biosciences) to select live human melanoma cells and exclude mouse endothelial and hematopoietic cells. Antibody labelling was performed for 20 min on ice, followed by washing and centrifugation. Before sorting, cells were resuspended in staining medium (L15 medium containing bovine serum albumin (1 mg/mL), 1% penicillin/streptomycin, and 10mM HEPES, pH 7.4) containing 4'6-diamidino-2-phenylindole (DAPI; 5µg/mL; Sigma) to eliminate dead cells from sorting. Live human melanoma cells were isolated by flow cytometry by sorting cells that were positive for dsRed and HLA and negative for mouse CD45, CD31, Ter-119 and DAPI.

Transplantation of melanoma cells

After sorting, cells were counted and resuspended in DMEM media with 50% high-protein Matrigel (product 354248, Corning). For a standard metastasis assay, 100 cells, for patient-derived melanoma cells, or 50 for A375, were injected subcutaneously into the right flank of the mice. Tumor formation was evaluated by regular palpitation of the injection site and tumors were measured weekly until any tumor in the mouse cohort reached 2.5cm in its largest diameter. Mice were monitored weekly for signs of distress according to a standard body condition score or within 24hr of their tumors reaching 2.5 cm in diameter – whichever came first. These experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at Weill Cornell Medicine (protocol 2017-0033).

Bioluminescence imaging

Mice injected subcutaneously with melanoma cells expressing luciferase were monitored until tumors reached 2.5cm in diameter. For bioluminescent imaging, mice were injected intraperitoneally with 100µL of DPBS containing D-luciferin monopotassium salt (40µg/mL, Goldbio) 5 min before imaging, followed by general anesthesia with isoflurane 2 min before imaging. IVIS Imaging System 200 Series (Caliper Life Sciences) with Living Image Software was used with the exposure time set to 10 seconds. After imaging the whole body, mice were euthanized and individual organs were dissected and imaged. The bioluminescent signal was quantified with 'region of interest' measurement tools in Living Image (Perkin Elmer) software.
After imaging, tumors and organs were fixed in paraformaldehyde for histopathology or metastatic nodules were collected to perform protein and/or molecular analysis.

Circulating tumor cell analysis

During imaging described above, once mice were euthanized for organ dissection, blood was also collected by cardiac puncture with a syringe pretreated with citrate-dextrose solution (Sigma). Red blood cells were precipitated by Ficoll sedimentation according to manufacturer's instructions (Ficoll Paque Plus, GE Healthcare). Remaining cells were washed with HBSS (Invitrogen) before antibody staining as described above and flow cytometric analysis.

Flow cytometric analysis of oxidative stress

Cells were plated in low attachment plates (3D system) or in TC-treated plates and treated with a prooxidant pyocyanin (Cayman). We stained the dissociated cells for 30 minutes at 37°C with 5 μM CellROX Green, or CellROX DeepRed in HBSS-free (Ca2+ and Mg2+ free) to assess mitochondrial and cytoplasmic ROS.

NADPH/NADP$^+$ measurement

For the mice results, subcutaneous tumors or metastatic nodules were surgically excised as quickly as possible after euthanizing the mice then melanoma cells were mechanically dissociated. For the melanoma cell lines, cell were plated the day before in 24 wells TC-treated plates. NADPH, NADP$^{+,}$ NADH and NAD$^+$ were measured using NADPH/NADP$^+$ Glo-Assay (Promega) and NADH/NAD$^+$ Glo-Assay (Promega) following the manufactures instructions. Luminescence was measured using a Omega plate reader (BMG Labtech FLUOstar). Values were normalized to protein concentration, measured using The Pierce Rapid Gold BCA Protein Assay Kit (Thermo Fisher Scientific).

Western Blot Analysis

Tissue or cell lines lysates were prepared using Triton lysis Buffer (Triton X-100 1%, 20mM Tris pH8, 137mM NaCl, 1mM EDTA, 10% glycerol, 1.5 mM $MgCl_2$) supplemented with phenylmethylsulphonyl fluoride (Sigma-Aldrich), and 2 cocktails of protease and phosphatase inhibitor (Halt™, Fisher Scientific; Phosstop™- phosphatase inhibitor tablets, Roche). The Pierce Rapid Gold BCA Protein Assay Kit (Thermo Fisher Scientific) was used to quantify protein concentrations. Equal amounts of protein (15–30 μg) were separated on 4–20% Tris Glycine SDS gels (BioRad) and transferred to polyvinylidene difluoride membranes (BioRad). Membranes were blocked for 30 minutes at room temperature with 5% milk in TBS supplemented with 0.1% Tween20 (TBST) then incubated with primary antibodies overnight at 4°C. After incubating with horseradish peroxidase conjugated secondary antibodies (Cell Signaling Technology), membranes were developed using SuperSignal West Pico or Femto chemiluminescence reagents (Thermo Fisher Scientific). The following primary antibodies were used for western blot analyses: NADK (Cell Signaling Technologies; 55948S), NADK (Sigma-Aldrich; HPA048909), NADK2 (Abcam; ab181028), FLAG M2 (Sigma-Aldrich; A2220), β-Actin HRP Conjugate, (Cell Signaling Technologies; 12262).

Cell culture

Melanoma cell line A375 was culture using DMEM (Corning; 10-017-CV) supplemented with 10% FBS and Penicillin: Streptomycin solution 100x (Corning; 45000-652).

Statistical analysis

No statistical methods were used to predetermine sample size. The data in most figure panels represents several independent experiments performed on different days. Variation is always indicated using standard deviation. For analysis of statistical significance, we first tested whether there was homogeneity of variation across conditions (as required by ANOVA) using Levene's test, or when only two conditions were compared, using the F-test. In cases where the variation was significantly different among conditions, we used a non-parametric Kruskal-Wallis test or a non-parametric Mann-Whitney test to assess significance of difference among populations and conditions. Usually variation did not significantly differ among conditions. Under those circumstances, two-tailed Student's t-tests were used to test significance of differences between two conditions. When more than one conditions were compared, a one-way ANOVA followed by Dunnett's multiple comparisons test was performed. A two-way ANOVA followed by Dunnett's multiple comparisons test were used in cases where more than two groups were compared with repeated measures. In all xenograft assays, we injected 5-8 week old NSG mice, 5 per condition. Both male and female mice were used. When mice died before the end of the experiment due to opportunistic infections the data from those mice was excluded.

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