SOX2-mediated upregulation of CD24 promotes adaptive resistance towards targeted therapy in melanoma

Laura Hüser[1], Sachindra[1], Karol Granados[1], Aniello Federico[1], Lionel Larribère[1], Daniel Novak[1], Viktor Umansky[1], Peter Altevogt[1] and Jochen Utikal[1]

[1] Skin Cancer Unit, German Cancer Research Center (DKFZ), Heidelberg and Department of Dermatology, Venereology and Allergology, University Medical Center Mannheim, Ruprecht-Karl University of Heidelberg, D-68135 Mannheim, Germany

Running head:

SOX2 and CD24 in melanoma

Key words: SOX2, CD24, BRAF inhibitor, adaptive resistance

Correspondence: Prof. Dr. Jochen Utikal, Skin Cancer Unit, German Cancer Research Center (DKFZ), and Department of Dermatology, Venereology and Allergology, University Medical Center Mannheim, Ruprecht-Karl University of Heidelberg, Heidelberg, Germany;
E-mail: j.utikal@dkfz.de, phone: 01149-621-383-4461

The authors declare no potential conflicts of interest

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1002/ijc.31609

Abbreviations: BRAFi: BRAF inhibitor; **ERK:** extracellular-signal regulated kinases; **EV**: empty vector; **IHC**: immunohistochemistry; **miRNA**: micro RNA; **mRNA:** messenger RNA; **ms:** mouse; **KD:** knock-down; **NFκB:** nuclear factor kappa B; **iPCC:** induced pluripotent cancer cell; **OE:** overexpression; **shRNA:** short hairpin RNA; **siRNA:** small interfering RNA; **rb**: rabbit; **STAT3:** signal transducer and activator of transcription 3; **TMA**: tissue microarray; **vem:** vemurafenib

Article Category: Molecular Cancer Biology

Novelty and Impact:

BRAF inhibitors are established in melanoma treatment but resistance is a major problem. Here we present a novel view how adaptive resistance is established. We show that BRAF inhibitor treatment leads to early STAT3 activation and promotes the expression of SOX2. SOX2 then binds to the CD24 promoter resulting in an upregulation of CD24 leading to an increased activity of Src and STAT3. This mechanism helps tumor cells to circumvent the action of BRAF inhibitors.

Summary

Melanoma is often characterized by a constitutively active RAS-RAF-MEK-ERK pathway. For targeted therapy, BRAF inhibitors are available that are powerful in the beginning but resistance occurs rather fast. A better understanding of the mechanisms of resistance is urgently needed to increase the success of the treatment. Here, we observed that SOX2 and CD24 are upregulated upon BRAF inhibitor treatment. A similar upregulation was seen in targeted therapy-resistant, melanoma-derived induced pluripotent cancer cells (iPCCs). SOX2 and CD24 are known to promote an undifferentiated and cancer stem cell-like phenotype associated with resistance. We, therefore, elucidated the role of SOX2 and CD24 in targeted therapy resistance in more detail. We found that the upregulation of SOX2 and CD24 required activation of STAT3 and that SOX2 induced the expression of CD24 by binding to its promoter. We find that the overexpression of SOX2 or CD24 significantly increases the resistance towards BRAF inhibitors, while SOX2 knock-down rendered cells more sensitivity towards treatment. The overexpression of CD24 or SOX2 induced Src and STAT3 activity. Importantly, by either CD24 knock-down or Src/STAT3 inhibition in resistant SOX2-overexpressing cells, the sensitivity towards BRAF inhibitors was re-established. Hence, we suggest a novel mechanism of adaptive resistance whereby BRAF inhibition is circumvented via the activation of STAT3, SOX2 and CD24. Thus, to prevent adaptive resistance it might be beneficial to combine Src/STAT3 inhibitors together with MAPK pathway inhibitors.

Introduction

Melanoma is a skin cancer that originates from melanocytes and can metastasize to different body parts. Although malignant melanoma just represents 4 % of all skin cancer cases, it is responsible for 80 % of deaths caused by skin cancer [1]. These facts demonstrate the aggressiveness of this cancer type and underline the importance for further studies aiming to find potent treatments. The most common mutations in melanoma are $BRAF^{V600E}$ (35-50%) or various NRAS mutations (appr. 20%) that lead to constitutive activation of the RAS-RAF-MEK-ERK pathway [2]. Therefore, BRAF inhibitors (BRAFis) such as vemurafenib (vem) were developed. These inhibitors initially show a remarkable effect, but resistance appears more or less shortly after the start of treatment [3]. In addition, around 15 % of melanoma patients have an intrinsic resistance towards vem and do not respond to vem treatment from the beginning on [4]. It is important to distinguish between acquired and adaptive resistance which both occur during vem treatment. Acquired resistance develops during continued BRAFi treatment, while adaptive resistance is the reaction of the tumor promptly after application of the drug [5].

Recently, induced pluripotent cancer cells (iPCCs) were established and it was shown that these cells have a higher resistance towards the MAP kinase pathway inhibitors vem and trametinib [6]. As these cells could represent a model for adaptive resistance, studying them could shed new light on the underlying mechanism of resistance. Since SOX2, one of the factors used to reprogram cancer cells towards iPCCs, favors a more undifferentiated phenotype [7], which has been shown to be associated with adaptive resistance [8, 9], SOX2 might play an important role in adaptive resistance. Several studies on the role of SOX2 in cancer have already been published, for example, it has been demonstrated that SOX2 plays a role in cancer cell stemness and invasion[10-12]. Studies in mice have however shown that

SOX2 is not required for melanomagenesis, melanoma growth and melanoma metastasis in vivo [13].

Another molecule we found to be upregulated in iPCCs was CD24. CD24 is a highly glycosylated protein with a molecular weight of 30-70 kDa that is linked to the membrane via a glycosyl-phosphatidylinositol (GPI) anchor. In humans, the protein core of CD24 comprises only 31 amino acids with many potential O- and N-glycosylation sites showing mucin-like characteristics [14]. CD24 is frequently overexpressed in human carcinomas [14, 15] and malignant melanomas [16, 17] and its expression is generally associated with a poor prognosis. In terms of biological function, CD24 has been identified as a ligand of P-selectin that supports the rolling of breast carcinoma cells on endothelial cells and adhesion to platelets [18, 19]. In addition, CD24 glycans can interact with siglecs, a class of sialic acid binding receptors on immune cells [15, 20]. CD24 expression can regulate tumor cell proliferation, adhesion, migration and invasion and alter gene expression in colon and pancreatic cancer cell lines [21].

Due to its GPI anchor, CD24 is exclusively localized in detergent-resistant membrane domains (DRMs) that have been also termed lipid rafts. These membrane microdomains are considered as important platforms for signaling molecules such as Src family tyrosine kinases and G-proteins. Indeed, several reports have shown that CD24 physically interacts and signals via members of the Src-kinase family [22-25].

Here we show that CD24 is upregulated in human melanoma cells upon SOX2 overexpression or treatment with vem. We identified the CD24-mediated activation of Src and STAT3 signalling as a novel pathway to establish resistance towards target therapy with BRAFi in melanoma. Our results strongly advocate the use of Src and

STAT3 inhibitors together with MAPK signalling inhibition to prevent the acquisition of adaptive resistance in melanoma.

Materials and Methods

Cell culture

The Human melanoma cell lines used in this study A375, SK Mel 28 and HT144 (all obtained from ATCC and $BRAF^{V600E}$ mutated) were cultured in DMEM (Gibco, Life Technologies) supplemented with 10 % heat-inactivated FBS (Biochrom), 0.1mM β-mercaptoethanol (Gibco, Life Technologies), 1% non-essential amino acids (NEAA) and 1% penicillin/streptomycin (Sigma-Aldrich). IPCCs were generated and cultured as described before [6]. All cell lines were cultured in a humidified incubator at 37°C and 5 % CO_2. Cell lines were sub-cultured every 3-5 days as soon as they reached around 80 % confluency.

Resistant cell lines

Resistant cells were generated by gradually increasing the concentration of the inhibitor in the culture medium. Hence only the resistant subpopulation continued growing. A375res cells (5 μM vem resistant cells) were kindly provided by Prof. Dr. David Proia (Synta Pharmaceuticals Corp., Lexington, Massachusetts) and SK Mel 28res cells were a kind gift from Prof. Dr. Joon Kim (Graduate School of Medical Science and Engineering, KAIST, Daejeon, Korea).

Cell viability assay

Melanoma cells were seeded in flat bottom 96-well plates (Gibco Life Technologies) at a density of 2500-5000 cells/well depending on the cell line and growth properties. At the following day, the cells were treated with different concentrations of vem (plx4032), plx8394 or plx7904 (all Selleck Chemicals), respectively, at the indicated

doses. To compare the sensitivity to vem with the sensitivity to a combination of vem and PP2 (Src kinase inhibitor dissolved in DMSO) or vem and BP-1-102 (STAT3 inhibitor dissolved in DMSO), 50 µM of PP2 or 13.5 µM of BP-1-102 50 µM of PP2 or the same volume of DMSO were added to the different concentration of vem. After 72h of treatment, Alamar blue (10 % of the culture medium volume) was added and after 3 ½ h of incubation at 37°C the fluorescence was measured at an emission wavelength of 535 nm and an excitation wavelength of 590 nm, using a SpectraMax M5 microplate reader (Tecan, Männedorf, Switzerland).

Inhibitor treatment

$2x10^5$ cells (for SK Mel 28 $5x10^4$ were used) were seeded in flat bottom 6-well plates (Gibco Life Technologies) and after attaching (within hours) they were treated with the inhibitor as indicated or the respective volume of solvent for control. The cells were incubated for 6 h, 24 h, 48 h, 72 h and 96 h and afterwards, RNA was isolated for qPCR analysis.

In case of the Src inhibitor PP2 (Selleckchem, Munich, Germany) the cells were seeded one day before the 72 h treatment with 50 µM PP2 or DMSO as control.

For the STAT3 inhibitor BP-1-102 (Merck Millipore) $1x10^6$ cells were seeded in a 10 cm dish one day prior to treatment. After 24h treatment with either DMSO, vem [3 µM], BP-1-102 [15 µM] or vem [3µM] + BP-1-102 [15 µM] or PP2 [50 µM] or PP2 [50 µM] + vem [3 µM] the cells were harvest and lysed. Dasatinib (Biovision) was used in the same experimental setup at a concentration of 100 nM.

Transduction with lentiviral particles

HEK293T cells were used for lentiviral particle production. HEK 293T cells were grown to approximately 60 % confluency before transfection. For transfection, 11 µg of the plasmid containing the gene/ shRNA of interest was mixed with the packaging plasmids VSV-G (5.5 µg) and pCMV-dR 8.91 (8.25 µg) in DMEM and X-treme

GENE® (Roche) solution. After 30 min of incubation at room temperature, the mixture of DNA, X-treme GENE and DMEM was added to HEK293T producer cells. After 12h, the supernatant was discarded. After another 12, 24, 36 h the supernatant containing the virus particles was collected and the virus particles were concentrated by ultracentrifugation. The concentrated virus was used to infect the melanoma cells. 24h after the first infection the melanoma cells were re-infected with the same virus in fresh medium and after 48h of transduction, the cells were washed twice with PBS and then cultured in their culturing medium. In order to select for transduced cells, cells were selected for 3 days in medium containing 0.8 µg/ml of puromycin or 15 µg/ml blasticidin. The following plasmids were used: Plasmid SOX2 OE: Plasmid #16577 (Addgene); Plasmids shRNA Plasmid shRNA SOX2: KD 1: TRCN0000231642; KD 2: TRCN0000257314; KD 3: TRCN0000355694; KD 4: TRCN0000231642; TRCN0000257314 TRCN0000355694 (Sigma); CD24 Plasmid: HsCD00418330 (Harvard Medical School, https://plasmid.med.harvard.edu/PLASMID/Home.xhtml).

Lipofectamine transfection

Cells were seeded to 60-80 % confluency and the siRNA transfection using Lipofectamine™ RNAiMAX Transfection Reagent (Thermo Fisher Scientific) was performed as per the manufacture's protocol. The cells were incubated for 72h before use in further experiments. siRNAs were obtained from Eurofins Genomics GmbH. The following siRNA were used according to [25]. Control siRNA GFP: 5'-GGC CAG GUC CAG CAG CGC ACC UU-3'; siRNA CD24: 5'-ACA ACA ACU GGA ACU UCA AUU-3'. Transduction efficacy was monitored by qPCR and Western Blot analysis.

Luciferase assay

CD24 promoter constructs pCD24-1896 and the respective pGL4.51 control vector were a kind gift of Dan Theodoresu (UC Denver, Colorando, USA). The renilla

plasmid pAAV psi2 [26] and the empty vector control pGL4.10 were a kind gift of Holger Sültmann (NCT, Heidelberg, Germany). In addition, the cells were co-transfected with the SOX2 OE construct (Addgene #16577) or empty vector as control. One day before transfection $5x10^3$ cells were seeded in flat bottom 96-well plates. At the next day, the cells were transfected with 100 ng total plasmid DNA. Thus, 66 ng of the firefly plasmids as well as 33 ng of the SOX2 OE or EV plasmid were transfected. Co-transfection with 1 ng renilla luciferase plasmid served as a transfection efficiency control. After 48h, firefly and renilla luciferase levels were determined by Dual-Glo® Luciferase Assay System (Promega) according to the manufacturer's instructions. Luciferase activity was normalized to renilla activity. Activities were calculated as the Relative Response Ratio (RRR) whereby the positive control pGL4.51 was 100 %.

Immunoprecipitation (IP)

Immunoprecipitation of CD24 was performed by lysing the cells of interest with 1x NP40 lysis buffer for 30 min on ice followed by 10 min full speed centrifugation. The antibody (SWA11, 5 µg) was added to the supernatant and incubated for 1h in a rotating wheel at 4 °C. Next, the antibody lysate mix was added to agarose G beads and incubated for 30 min at 4 °C under rotation. After washing the beads, sample buffer was added and the samples were cooked for 5 min at 95 °C. A western blot was run to visualize the result. To ensure an equal input, a small aliquot of the lysate was saved to serve as loading control.

Tissue microarray (TMA) staining

Tumor samples of melanoma patients were used to prepare TMA-slides as reported before [27]. Briefly, tissue punshes of metastatic melanoma patient samples were collected and fixed in formalin and afterwards embedded in paraffin to obtain a TMA. After cutting the TMA was overnight incubated with primary antibody. Next, the slides were washed in TBS-T and secondary antibody staining (Dako EnVisionTM +

System-HRP; AEC K4009) was performed according to the manufacture's protocol. The samples were counterstained with H&E and mounted (Dako S3025). To ensure that the analyzed regions contained tumor cells, the tumor cells were visualized by S100β. The sections were examined in a blinded setup by two individuals. As scoring critira the quantity and the intensity of the staining were used whereby a low score demonstrates a low quantity/ intensity and a high score a high quantity/ intensity of the staining. The following antibodies were used to stain the TMA-slides: SOX2rb (ab97959 Abcam) 1:100; CD24ms (Sn3b, Thermo Fisher scientific) 1:50.

Statistical analysis

All statistics were performed using GraphPad Prism (GraphPad Software) and statistical tests were performed on at least 3 independent experiments if not indicated differently. Only the microarray data were statistically analysed using Chipster.

Additional Methods

These additional Methods can be found in the supplement:

RNA isolation, cDNA synthesis, qPCR, Protein isolation, Westernblot, Fluorescence-activated cell sorting (FACS) and Microarray gene expression profiling

Results

SOX2 and CD24 is up-regulated in melanoma iPCCs and after treatment with MEK/BRAF inhibitors

In a previous study, we have shown that iPCCs generated from melanoma cells by stable reprogramming with the transcription factors OCT4, KLF4, and SOX2 are more resistant towards targeted therapy (MEK and BRAF inhibitors) than their parental counterpart [6]. Using microarray expression data, we observed that SOX2 and CD24 were significantly increased in iPCC-HT144 in comparison to the parental HT144 cells (Fig.1A). A high expression level of SOX2 was expected as it is a crucial pluripotency factor that is part of the iPCC vector used for the reprogramming of melanoma cells [6]. Importantly, when comparing this data set with genes changed after 24h of vem treatment of A375 cells, SOX2 and CD24 were also significantly upregulated (Fig.1B). Therefore, the role of SOX2 and CD24 in adaptive resistance became the focus of our work. Further experiments, in which A375, HT144 and SK Mel 28 cells were treated for various time periods, confirmed that the treatment with BRAFi led to a significant increase in SOX2 and CD24 expression as detected by RT-PCR analysis (Fig. 1C, D, and suppl. Fig.1A-D). Although mRNA levels were fluctuating over time, the vem treatment leads to a robust upregulation of SOX2 and CD24 at the protein level (Fig. 1E). Similar results were obtained with BRAFis plx8394 and plx7904 (suppl. Fig. 2A and B)

Additional experiments using cytofluorographic analysis and Annexin-V staining to detect apoptotic cells revealed that the vem-mediated upregulation of CD24 was due to induction of expression rather than the result of selective killing of CD24 negative cells (suppl. Fig. 3).

As the combination treatment with MEK and BRAF inhibitors is state of the art in

melanoma therapy, A375 cells were also treated with a combination of both drugs. Again, a significant increase in SOX2 and CD24 expression was noted at the mRNA level (suppl. Fig. 1E). Collectively, these data suggest that the induced expression of SOX2 and CD24 might play a role in adaptive resistance.

SOX2 regulates the expression of CD24

We next investigated the relationship between SOX2 and CD24 expression. For this, we overexpressed SOX2 in the melanoma cell lines A375 and SK Mel 28 (Fig. 2A and suppl. Fig 4A) and examined subsequently the level of CD24 expression. We observed a significant increase of CD24 expression on both mRNA and protein level upon SOX2 OE (Fig. 2B and suppl. Fig. 4B and C).

To corroborate these data in clinical samples from patients with metastatic melanoma, we analyzed a TMA of 60 tissues by IHC for SOX2 and CD24 expression. We noticed a significant positive correlation ($r=0.4$; $p= 0.004$) between SOX2 and CD24 expression (Fig.2C).

To investigate if there is a direct regulation of CD24 by SOX2 at the transcriptional level, we examined the CD24 promoter for possible SOX2 binding sites. Indeed, using the JASPAR prediction tool (http://jaspar.genereg.net) we identified 6 putative binding sites for SOX2 in the CD24 promoter as displayed in Fig. 2D. To examine if SOX2 directly binds to these sites, we made use of a CD24-promoter luciferase construct published elsewhere [28]. This construct comprises the whole promoter upstream of the luciferase gene as displayed in Fig. 2D. Transient SOX2 overexpression in HEK293T cells that were also transfected with the CD24 promoter-luciferase reporter plasmid showed strongly augmented luciferase activity compared to the same cells carrying an empty vector control plasmid (Fig. 2E and F). Similar

results albeit not significant due to the low transfection efficacy were obtained in A375 melanoma cells (Fig.2G and H).

Our results are consistent with published data on the overexpression of SOX2 in human embryonic stem cells and human skin fibroblasts [29, 30]. CHIP-Seq analysis 48 h post induction revealed binding of SOX2 to the CD24 promoter in position -1342 from the transcription start site [29, 30]. These additional results make it quite likely, that SOX2 activates CD24 expression by binding to this site indicated in Fig. 2D.

SOX2 or CD24 overexpression augments resistance towards vemurafenib and induces activation of Src and STAT3

We asked whether an increased expression level of SOX2 would alter the resistance towards vem treatment. Using A375 and SK Mel 28 melanoma cells, there was a significant increase in the IC50 value in SOX2-overexpressing cells compared to vector control cells (A375: EV mean IC50= 165 nM, SOX2 OE mean= 269 nM; p value= 0.045 (Fig.3 A) and SK Mel 28: EV mean IC50= 234 nM, SOX2 OE mean= 350 nM, p-value=0.02 (suppl. Fig. 4D)).

We hypothesized that CD24 might be involved in this process. Previous work in human carcinoma cells had shown that forced overexpression of CD24 increases activity of Src and STAT3 [25]. We argued that the SOX2-mediated upregulation of CD24 might cause a similar effect in melanoma cells. Indeed, upon SOX2 OE in A375 cells (Fig. 3C) or SK Mel 28 (suppl. Fig. 4C) we observed an increased phosphorylation of Src, STAT3 and ERK that could be related to the increased resistance towards BRAFi. Importantly, OE of CD24 in A375 cells showed very similar results (Fig. 3B and D, suppl. Fig. 4E and F) whereas an activation of Src, but not STAT3, together with increased resistance to vem was also noted in SK Mel 28 cells (suppl. Fig. 4G and H).

Importantly, the Src kinase inhibitor PP2 lead to a significant decrease of the IC50 values in the more vem-resistant SOX2 or CD24 OE A375 cells (Fig. 3E, right panel). Noteworthy, PP2 blocked the phosphorylation of Src but did not affect the phosphorylation of STAT3 (Fig.3E, left panel). Similar results were obtained when dasatinib instead of PP2 was used (suppl. Fig 5).

In contrast, the vem-induced phosphorylation of STAT3 was blocked in the presence of the STAT3-specific inhibitor BP-1-102 but was not affected in the presence of PP2 (Fig. 3F left panel). Similar to Src inhibition, the inhibition of STAT3 using BP-1-102 decreased the IC50 value in the more vem-resistant SOX2 or CD24 OE A375 cells (Fig. 3F right panel).

Thus, the results obtained here suggest that the activation of STAT3 is not dependent on Src phosphorylation. The data are also compatible with previous findings demonstrating that the inhibition of Src can overcome BRAFi resistance [31, 32]. Collectively, our results indicate that SOX2 plays a role in the resistance towards vem by augmenting the activity of the CD24-Src/STAT3 signalling pathway.

SOX2 or CD24 knock-down result in a higher sensitivity towards BRAFis

To further analyze the impact of SOX2 in cell viability and BRAFi resistance, we stably KD its expression via shRNA and several clones (KD1-4) showing SOX2 depletion were established (Fig. 4A and suppl. Fig.7A and B). SOX2 KD in A375 (Fig. 4B), Sk Mel 28 (suppl. Fig. 7A) or HT144 (suppl. Fig. 7B) that all represent BRAF mutant cell lines, showed a higher sensitivity towards BRAFis with a lower IC50 value than the respective empty vector control cells. In addition, we examined the effect of CD24 KD on cell viability. As in A375 as well as SK Mel 28 cells the endogenous expression of CD24 is very low we used A375 SOX2-overexpressing cells which showed a high expression of CD24 (suppl. Fig. 6) to perform CD24 KD experiments.

We found that the KD of CD24 in SOX2-overexpressing cells resulted in an increased sensitivity towards BRAFis (Fig. 4C and suppl. Fig. 7C). The corresponding viability curves to all viability experiments are shown in suppl. Fig. 8.

Furthermore, the KD of CD24 in A375 SOX2-overexpressing cells showed a reduced activation of Src and STAT3 confirming earlier results in carcinoma cells [25] also in melanoma cells (Fig. 4D). Taken together, these results suggest that in melanoma cells SOX2 and CD24 play an important role in conferring BRAFi resistance via regulating Src and STAT3 activity.

STAT3 activation is required for SOX2 induction

STAT3 is a transcription factor that following phosphorylation at tyrosine 705 dimerizes and translocates to the nucleus, where it binds to target genes and promotes transcription [33]. We asked whether STAT3 was involved in the induction of SOX2 by vem treatment. Indeed, we noticed that the phosphorylation of STAT3 was an early event and detectable already 6 h after onset of treatment (Fig. 5A). In the presence of the STAT3 inhibitor BP-1-102 the vem-induced upregulation of CD24 and SOX2 were diminished on the protein level (Fig.5B) and on the mRNA level (Fig. 5C and D). Our data clearly suggested, that STAT3 activation is required for the induction of SOX2 expression in A375 melanoma cells. A similar regulation of SOX2 by STAT3 in neural precursor cells [34] and the binding of STAT3 to the SOX2 promoter in melanoma cells were shown before [35].

SOX2 has no major role in acquired therapy resistance

To examine whether SOX2 plays a role in acquired resistance, the SOX2 expression in cell lines showing established resistance (A375 res, HT144 res and SK Mel28 res cells) to targeted therapy was analyzed. We observed that SOX2 expression was not

permanently upregulated in the resistant cell lines (suppl. Fig. 9 A-C). Only in one cell line (HT144 res), there was increased expression of SOX2. While in most of the cell lines SOX2 expression remained unchanged, in resistant SK Mel 28 cells it was even downregulated. We also did not observe any correlation between SOX2 and CD24 expression in resistant cell lines (data not shown).

Much in contrast, ERK phosphorylation was high in all resistant cell lines (suppl. Fig. 8D). Previous findings by others also showed that SOX2 was unchanged in BRAFi resistant cell lines [13]. Overall, these data demonstrate that SOX2 is most likely important in adaptive but not in acquired resistance.

Discussion

In the present work we show that i) SOX2 and CD24 were upregulated upon short-term BRAFi treatment in melanoma cells and a similar upregulation was observed in targeted therapy resistant iPCCs; ii) a positive correlation between SOX2 and CD24 expression was observed by IHC in tissue sections of metastatic melanoma specimens; iii) SOX2 could directly bind and activate the CD24 promoter as revealed in luciferase reporter assays; iv) overexpression of SOX2 led to an increase in resistance towards BRAFi which was prevented by CD24 KD; v) CD24 overexpression could mimic the effect of SOX2 overexpression; vi) SOX2 KD resulted in a similarly decreased resistance towards BRAFis; vii) activated STAT3 is required for the induction of SOX2 expression forming a feedback loop. We suggest that the regulation of Src and STAT3 activity by SOX2 via CD24 is a novel mechanism of adaptive resistance towards BRAFis in melanoma cells. Our findings are summarized as a model in Fig. 6.

Several studies have shown that CD24 and SOX2 are associated with a more undifferentiated and more cancer stem cell-like phenotype [7, 36, 37]. Both SOX2 and CD24 have also been linked to an increased therapy resistance [38-41]. This is in line with our data demonstrating that in melanoma cells an increased expression level of SOX2 or CD24 resulted in a higher tolerance towards BRAFis, while the depletion of SOX2 via KD showed higher sensitivity. Similarly, the KD of CD24 in the more drug resistant SOX2 OE cells led to a restoration of sensitivity. These findings indicate that SOX2 expression induced a higher resistance towards BRAFis via the upregulation of CD24, since overexpression of CD24 showed the same effect as SOX2 overexpression. Previous work had demonstrated that CD24 has an influence on oncological properties such as cell proliferation and survival via an increased activity of Src and STAT3 [24, 25]. We, therefore, speculated that the upregulation of Src and

STAT3 activity might be the reason for the SOX2/ CD24 associated resistance. Indeed, cells overexpressing SOX2 showed a higher activity of Src and STAT3. These results are in good agreement with the study of Wang et al. showing that SOX2 regulates the activity of Src in ovarian cancer [42]. In addition, we could show that Src inhibition could overcome the SOX2-dependent resistance towards vem. This is consistent with recent studies showing that Src inhibition can overcome resistance by Girotti et al. [31, 32]. Furthermore, we could show that a KD of CD24 resulted in a decreased activity of Src and STAT3 and CD24 overexpression activated Src and STAT3, confirming the study of Bretz et al. [25] for malignant melanoma. Interestingly, several studies [43-45] showed that STAT3 activation plays an important role in resistance. Lee et al. observed that CD24 drives tumor initiation via STAT3 [44] and Wang et al. showed that CD24 can regulate the MAPK pathway, demonstrating the importance of CD24 in tumor cell survival and proliferation [46]. Additionally, we could show that more resistant SOX2 and CD24 OE cells are still sensitive to STAT3 inhibition. This is in good agreement with the study of Liu et al. [43] demonstrating that STAT3 inhibition can overcome resistance towards targeted therapies.

Are the activation of Src and STAT3 interrelated? Bretz et al. have shown in carcinoma cells that CD24 KD impaired STAT3 phosphorylation and similar results were obtained when Src was depleted [25]. The authors suggested that Src was the kinase linking CD24 to the activation of STAT3. Similar findings were presented by Niu et al. for malignant melanoma [47]. In contrast, in our experiments, we did not observe any effect of the Src kinase inhibitors PP2 or dasatinib on the vem-mediated phosphorylation of STAT3. Likewise, in A375 SOX2 OE cells that showed strong STAT3 activation the PP2 treatment did not alter this status. In addition, we could observe an upregulation of STAT3 already after 6 h of vem treatment while Src was

only activated after 96 h of vem treatment (data not shown). Interestingly, in SK Mel 28 cells the OE of CD24 leads to the activation of Src but not of STAT3 (suppl. Fig. 4G). Thus, we conclude that in our melanoma cell line model Src is not the kinase driving activation of STAT3. A recent study on colorectal cancer has shown that Hsp90 can serve as a signal-transmitting link between CD24 and STAT3 [48].

We show that early activation of STAT3 is required to obtain an increased expression of SOX2. This initial STAT3 activation might be due to secreted factors as suggested by Ohanna et al. showing that the secretome of senescent melanoma cells drives basal melanoma cells towards a mesenchymal phenotype and activates the STAT3 pathway [49]. Pietrobono showed that Gentian violet (GV), a cationic triphenylmethane can repress melanoma stem cell self-renewal through inhibition of SOX2 [35]. The authors suggested that GV suppressed STAT3 activation through an EGFR-dependent mechanism [35]. Thus, it is quite possible that diverse signalling pathways can lead to STAT3 activation which in turn can trigger SOX2 expression. It is becoming clear that feedback activation of STAT3 plays a prominent role in mediating drug resistance to a broad spectrum of targeted cancer therapies and chemotherapies including melanoma.

A recent study by Fallahi-Sichani has shed some new light on the cellular response to vem in melanoma [8]. It was shown on a single-cell level that the exposure of tumor cells to BRAFi caused a heterogeneous response in which some cells die, some arrest, and the remainder adapt to the drug. Drug-adapted cells upregulate markers of the neural crest (e.g., NGFR), a melanocyte precursor, and grow slowly. Additional analysis showed that drugs targeting the c-Jun/ECM/FAK/Src cascade can increase the maximum effect of BRAFi by promoting cell killing. Thus, the analysis at the single-cell level identifies signaling pathways and inhibitory drugs missed by assays

that focus on cell populations [8].

Finally, a limitation of our study is that SOX2 apparently does not play a major role in acquired resistance. This conclusion is based on our observation that in the established vem-resistant cell lines HT144 res, A375 res and SK Mel 28 res SOX2 expression was not permanently upregulated and vem exposure could not upregulate SOX2 anymore. (suppl. Fig. 9). In contrast, ERK phosphorylation was high in all resistant cell lines. Our observations are in agreement with a recent publication demonstrating that SOX2 has no oncogenic function during melanoma development and is not required for the acquisition of resistance to BRAFi treatments [13]. Therefore, we conclude that SOX2-mediated upregulation of CD24 and the activation of Src and STAT3 are only instrumental in establishing resistance shortly after the start of vem treatment. It most likely acts as an emergency plan to rescue many cancer cells from death induced by BRAFi treatment.

Taken together, our work helps to dissect the various steps involved in vem-resistance induction, focusing on adaptive resistance. We suggest that BRAFi treatment leads to an increase in STAT3 activation shortly after treatment which promotes SOX2 expression. The increase in SOX2 expression further upregulates the expression of CD24 that activates Src/STAT3 pathways leading to increased cell survival. Hence, to prevent adaptive resistance in clinical settings it might be beneficial to use Src or STAT3 inhibitors together with MAPK pathway inhibitors in order to block this escape pathway. Another option might be to target CD24 with mAb which has been shown to result in decrease Src and STAT3 activation in xenograft tumor models[50].

Acknowledgements

This work is part of the PhD thesis of Laura Hüser. We would like to acknowledge Sayran Arif-Said and Jennifer Dworacek for their excellent technical assistance. We wish to thank Dan Theodorescu (University of Denver, CO) for the kind gift of CD24 promoter constructs and the DKFZ Genomics and Proteomics Core Facility for competent help. We would like to thank the Tissue Bank of the National Center for Tumor Diseases for TMA preparation and automated slide scanning. This work was supported by a grant of the German Research Council RTG2099 ”Hallmarks of skin cancer”.

Legends to Figures

Figure 1: SOX2 and CD24 are highly upregulated in BRAFi resistant melanoma iPCCs and in short-time BRAFi treated melanoma cells

A: Microarray data from iPC-HT144 cells in comparison to the parental HT144 cells show a significant upregulation of SOX2 and CD24 in the iPCCs (fold change in log2 scale). Next 2 graphs show validation of microarray data by qPCR. qPCR data are shown as the relative expression level of mRNA, normalized to 18S (n=2). **B:** Microarray data from A375 treated for 24h with 3 μM vem or the corresponding amount of DMSO show significant upregulation of SOX2 and CD24 after vem treatment. **C:** SOX2 is significantly upregulated after different time periods of BRAFi (vem, plx8394, plx7904) treatment [3 μM] in A375 cells. **D:** CD24 is significantly unregulated after treatment with BRAFi in A375 cells. **E:** The Expression of SOX2 and CD24 under vem treatment was analysed by western blot at different time points as indicated. SOX2 and CD24 expression is highly increased after vem treatment. ($p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***)

Figure 2: CD24 is regulated by SOX2 and their levels are correlated in metastatic melanoma patient samples

A: Validation of SOX2 overexpression (OE) in A375 cells by qPCR showing relative expression level of mRNA, normalized to 18S and on protein level in WB. EV= empty vector **B:** qPCR and WB demonstrating an increased expression of CD24 in A375 SOX2 OE cells. **C:** Left: IHC staining of a TMA of metastatic melanoma stained for SOX2, CD24 and S100β as indicated. The inlay shows the same sample at a higher magnification. The top row shows an example for a low score of SOX2 and CD24

while the sample for sure contains tumor cells as demonstrated by S100β. The lower row shows a TMA sample highly positive for SOX2 and CD24 while S100β demonstrate the presents of tumor cells. Right: Pearson's correlation analysis of CD24 and SOX2 IHC reveal positive correlation of SOX2 and CD24 expression levels in metastatic melanoma samples of patients. **D**: Schematic drawing of CD24 promotor with its potential SOX2 binding sites followed by the luciferase reporter as it is used in the luciferase assay. The most likely binding site due to ChIPseq data is circled. The plasmids were transiently transfected into HEK and A375 cells either together with a SOX2 OE plasmid or an EV control for 48 h. The CMV promotor served as positive control and the pGL4.10 empty vector was used as negative control. **E:** Transient OE of SOX2 in HEK cells validated by qPCR. **F:** The luciferase activity dependent on the SOX2 expression level was measured and all were normalized to the *Renilla* luciferase activity. The Relative Response Ratio (RRR) is significantly higher in the SOX2-overexpressing cells. **G:** Transient SOX2 OE in A375 cells as validated by qPCR. **H:** The RRR is slightly increased in A375 cells overexpressing SOX2 in comparison to EV control cells. ($p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***)

Figure 3: Upregulation of SOX2 or CD24 goes along with increased resistance towards BRAFi and activation of Src, STAT3 and ERK

A: A375 SOX2 OE cells show a higher IC50 than empty vector (EV) control cells and are therefore less sensitive towards vem. **B**: A375 CD24 OE cells show a higher IC50 when compared to EV control cells. Therefore these cells can tolerate a higher concentration of vem **C:** WB showing an increased activation of Src, STAT3, and

ERK in SOX2-overexpressing A375 cells. **D:** CD24 overexpression in A375 results in an increased activation of Src, STAT3 and ERK. **E:** A375 SOX2 OE cells were stimulated with PP2 [50 μM], an inhibitor of Src. WB showing Src inactivation but no changes in STAT3 activation. Treatment of A375 SOX2 OE or A375 CD24 OE cells with PP2 [50 μM] in addition to vem leads to a decreased IC50. **F:** Left: STAT3 is activated by vem [3 μM] and can be inhibited by BP-1-102 [15 μM] but not by PP2 [50 μM]. Right: the more resistant A375 SOX2 OE/ CD24 OE cells can still be killed by the STAT3 inhibitor BP-1-102 as demostrated by the lower IC50 value of the in addition to vem with BP-1-102 treated cells. ($p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***)

Figure 4: KD of SOX2 or CD24 causes a higher sensitivity towards BRAFi treatment

A: WB demonstrating that the shRNA-mediated KD of SOX2 in A375 cells is effective. **B:** A375 cells with a KD of SOX2 show a higher sensitivity towards vem. **C:** siRNA KD of CD24 in A375 SOX2 OE cells results in a decreased resistance towards vem as shown by the lower IC50 in the CD24 KD cells. **D**: siRNA KD of CD24 leads to a decreased activation of Src and STAT3 in A375 SOX2 OE cells. ($p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***)

Figure 5: STAT3 activation by vem is essential for increased SOX2 and CD24 expression

A: WB of A375 treated for the indicated times with 3 μM vem or DMSO as control. The STAT3 activation is depicted by pSTAT as well as the total STAT3 level as detected by WB as shown. **B:** WB of A375 treated for 24h with DMSO, vem [3 μM], BP-1-102 [15 μM], vem [3 μM] + BP-1-102 [15 μM]. The WB demonstrats the

increased STAT3 activation upon vem treatment can be inhibited in the presence of the STAT3 inhibitor BP-1-102. Furthermore, the vem induced SOX2 and CD24 expression is reduced if the STAT3 inhibitor is present in addition to vem. **C/D:** qPCR data which confirming the WB results for SOX2 and CD24 on the mRNA level. qPCR showing relative expression level of mRNA, normalized to 18S. ($p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***)

Figure 6: Schematic overview of the SOX2 and CD24-mediated adaptive resistance

The treatment with BRAFi leads to an activation of STAT3 that induces a higher expression of SOX2. SOX2 promotes the expression of CD24 by binding to its promotor. The increased CD24 expression causes a higher Src and STAT3 activation contributing to cell survival. STAT3 may act as an autocrine stimulus for further SOX2 expression.

References

1. Miller AJ, Mihm MC, Jr. Melanoma. The New England journal of medicine 2006;355:51-65.

2. Cancer Genome Atlas N. Genomic Classification of Cutaneous Melanoma. Cell 2015;161:1681-96.

3. Stadler S, Weina K, Gebhardt C, Utikal J. New therapeutic options for advanced non-resectable malignant melanoma. Advances in medical sciences 2015;60:83-8.

4. Spagnolo F, Ghiorzo P, Orgiano L, Pastorino L, Picasso V, Tornari E, Ottaviano V, Queirolo P. BRAF-mutant melanoma: treatment approaches, resistance mechanisms, and diagnostic strategies. OncoTargets and therapy 2015;8:157-68.

5. Kugel CH, 3rd, Aplin AE. Adaptive resistance to RAF inhibitors in melanoma. Pigment cell & melanoma research 2014;27:1032-8.

6. Bernhardt M, Novak D, Assenov Y, Orouji E, Knappe N, Weina K, Reith M, Larribere L, Gebhardt C, Plass C, Umansky V, Utikal J. Melanoma-Derived iPCCs Show Differential Tumorigenicity and Therapy Response. Stem cell reports 2017;8:1379-91.

7. Zhang S, Cui W. Sox2, a key factor in the regulation of pluripotency and neural differentiation. World journal of stem cells 2014;6:305-11.

8. Fallahi-Sichani M, Becker V, Izar B, Baker GJ, Lin JR, Boswell SA, Shah P, Rotem A, Garraway LA, Sorger PK. Adaptive resistance of melanoma cells to RAF inhibition via reversible induction of a slowly dividing de-differentiated state. Molecular systems biology 2017;13:905.

9. Sachindra, Larribere L, Novak D, Wu H, Huser L, Granados K, Orouji E, Utikal J. New role of ID3 in melanoma adaptive drug-resistance. Oncotarget 2017;8:110166-75.

10. Bernhardt M, Galach M, Novak D, Utikal J. Mediators of induced pluripotency and their role in cancer cells - current scientific knowledge and future perspectives. Biotechnology journal 2012;7:810-21.

11. Weina K, Utikal J. SOX2 and cancer: current research and its implications in the clinic. Clinical and translational medicine 2014;3:19.

12. Weina K, Wu H, Knappe N, Orouji E, Novak D, Bernhardt M, Huser L, Larribere L, Umansky V, Gebhardt C, Utikal J. TGF-beta induces SOX2 expression in

a time-dependent manner in human melanoma cells. Pigment cell & melanoma research 2016;29:453-8.

13. Cesarini V, Guida E, Todaro F, Di Agostino S, Tassinari V, Nicolis S, Favaro R, Caporali S, Lacal PM, Botti E, Costanzo A, Rossi P, et al. Sox2 is not required for melanomagenesis, melanoma growth and melanoma metastasis in vivo. Oncogene 2017;36:4508-15.

14. Kristiansen G, Sammar M, Altevogt P. Tumour biological aspects of CD24, a mucin-like adhesion molecule. J Mol Histol 2004;35:255-62.

15. Kristiansen G, Machado E, Bretz N, Rupp C, Winzer KJ, Konig AK, Moldenhauer G, Marme F, Costa J, Altevogt P. Molecular and clinical dissection of CD24 antibody specificity by a comprehensive comparative analysis. Lab Invest 2010;90:1102-16.

16. Tang MR, Wang YX, Guo S, Han SY, Li HH, Jin SF. CD24 expression predicts poor prognosis for patients with cutaneous malignant melanoma. International journal of clinical and experimental medicine 2014;7:4337-41.

17. Gschaider M, Neumann F, Peters B, Lenz F, Cibena M, Goiser M, Wolf I, Wenzel J, Mauch C, Schreiner W, Wagner SN. An attempt at a molecular prediction of metastasis in patients with primary cutaneous melanoma. PloS one 2012;7:e49865.

18. Aigner S, Sthoeger ZM, Fogel M, Weber E, Zarn J, Ruppert M, Zeller Y, Vestweber D, Stahel R, Sammar M, Altevogt P. CD24, a mucin-type glycoprotein, is a ligand for P-selectin on human tumor cells. Blood 1997;89:3385-95.

19. Aigner S, Ramos CL, Hafezi-Moghadam A, Lawrence MB, Friederichs J, Altevogt P, Ley K. CD24 mediates rolling of breast carcinoma cells on P-selectin. Faseb J 1998;12:1241-51.

20. Chen GY, Tang J, Zheng P, Liu Y. CD24 and Siglec-10 selectively repress tissue damage-induced immune responses. Science 2009;323:1722-5.

21. Sagiv E, Starr A, Rozovski U, Khosravi R, Altevogt P, Wang T, Arber N. Targeting CD24 for treatment of colorectal and pancreatic cancer by monoclonal antibodies or small interfering RNA. Cancer Res 2008;68:2803-12.

22. Zarn JA, Zimmermann SM, Pass MK, Waibel R, Stahel RA. Association of CD24 with the kinase c-fgr in a small cell lung cancer cell line and with the kinase lyn in an erythroleukemia cell line. Biochem Biophys Res Commun 1996;225:384-91.

23. Sammar M, Gulbins E, Hilbert K, Lang F, Altevogt P. Mouse CD24 as a signaling molecule for integrin-mediated cell binding: functional and physical association with src-kinases. Biochem Biophys Res Commun 1997;234:330-4.

24. Baumann P, Thiele W, Cremers N, Muppala S, Krachulec J, Diefenbacher M, Kassel O, Mudduluru G, Allgayer H, Frame M, Sleeman JP. CD24 interacts with and promotes the activity of c-src within lipid rafts in breast cancer cells, thereby increasing integrin-dependent adhesion. Cellular and molecular life sciences : CMLS 2012;69:435-48.

25. Bretz NP, Salnikov AV, Perne C, Keller S, Wang X, Mierke CT, Fogel M, Erbe-Hofmann N, Schlange T, Moldenhauer G, Altevogt P. CD24 controls Src/STAT3 activity in human tumors. Cellular and molecular life sciences : CMLS 2012;69:3863-79.

26. Borner K, Niopek D, Cotugno G, Kaldenbach M, Pankert T, Willemsen J, Zhang X, Schurmann N, Mockenhaupt S, Serva A, Hiet MS, Wiedtke E, et al. Robust RNAi enhancement via human Argonaute-2 overexpression from plasmids, viral vectors and cell lines. Nucleic acids research 2013;41:e199.

27. Wagner NB, Weide B, Reith M, Tarnanidis K, Kehrel C, Lichtenberger R, Pflugfelder A, Herpel E, Eubel J, Ikenberg K, Busch C, Holland-Letz T, et al. Diminished levels of the soluble form of RAGE are related to poor survival in malignant melanoma. International journal of cancer 2015;137:2607-17.

28. Overdevest JB, Knubel KH, Duex JE, Thomas S, Nitz MD, Harding MA, Smith SC, Frierson HF, Conaway M, Theodorescu D. CD24 expression is important in male urothelial tumorigenesis and metastasis in mice and is androgen regulated. Proceedings of the National Academy of Sciences of the United States of America 2012;109:E3588-96.

29. Tsankov AM, Gu H, Akopian V, Ziller MJ, Donaghey J, Amit I, Gnirke A, Meissner A. Transcription factor binding dynamics during human ES cell differentiation. Nature 2015;518:344-9.

30. Soufi A, Donahue G, Zaret KS. Facilitators and impediments of the pluripotency reprogramming factors' initial engagement with the genome. Cell 2012;151:994-1004.

31. Girotti MR, Pedersen M, Sanchez-Laorden B, Viros A, Turajlic S, Niculescu-Duvaz D, Zambon A, Sinclair J, Hayes A, Gore M, Lorigan P, Springer C,

et al. Inhibiting EGF receptor or SRC family kinase signaling overcomes BRAF inhibitor resistance in melanoma. Cancer discovery 2013;3:158-67.

32. Girotti MR, Lopes F, Preece N, Niculescu-Duvaz D, Zambon A, Davies L, Whittaker S, Saturno G, Viros A, Pedersen M, Suijkerbuijk BM, Menard D, et al. Paradox-breaking RAF inhibitors that also target SRC are effective in drug-resistant BRAF mutant melanoma. Cancer cell 2015;27:85-96.

33. Darnell JE, Jr. STATs and gene regulation. Science 1997;277:1630-5.

34. Foshay KM, Gallicano GI. Regulation of Sox2 by STAT3 initiates commitment to the neural precursor cell fate. Stem cells and development 2008;17:269-78.

35. Pietrobono S, Morandi A, Gagliardi S, Gerlini G, Borgognoni L, Chiarugi P, Arbiser JL, Stecca B. Down-Regulation of SOX2 Underlies the Inhibitory Effects of the Triphenylmethane Gentian Violet on Melanoma Cell Self-Renewal and Survival. The Journal of investigative dermatology 2016;136:2059-69.

36. Lundberg IV, Edin S, Eklof V, Oberg A, Palmqvist R, Wikberg ML. SOX2 expression is associated with a cancer stem cell state and down-regulation of CDX2 in colorectal cancer. BMC cancer 2016;16:471.

37. Yang CH, Wang HL, Lin YS, Kumar KP, Lin HC, Chang CJ, Lu CC, Huang TT, Martel J, Ojcius DM, Chang YS, Young JD, et al. Identification of CD24 as a cancer stem cell marker in human nasopharyngeal carcinoma. PloS one 2014;9:e99412.

38. Song WS, Yang YP, Huang CS, Lu KH, Liu WH, Wu WW, Lee YY, Lo WL, Lee SD, Chen YW, Huang PI, Chen MT. Sox2, a stemness gene, regulates tumor-initiating and drug-resistant properties in CD133-positive glioblastoma stem cells. Journal of the Chinese Medical Association : JCMA 2016;79:538-45.

39. Piva M, Domenici G, Iriondo O, Rabano M, Simoes BM, Comaills V, Barredo I, Lopez-Ruiz JA, Zabalza I, Kypta R, Vivanco M. Sox2 promotes tamoxifen resistance in breast cancer cells. EMBO molecular medicine 2014;6:66-79.

40. Surowiak P, Materna V, Paluchowski P, Matkowski R, Wojnar A, Maciejczyk A, Pudelko M, Kornafel J, Dietel M, Kristiansen G, Lage H, Zabel M. CD24 expression is specific for tamoxifen-resistant ductal breast cancer cases. Anticancer research 2006;26:629-34.

41. Deng X, Apple S, Zhao H, Song J, Lee M, Luo W, Wu X, Chung D, Pietras RJ, Chang HR. CD24 Expression and differential resistance to chemotherapy in triple-negative breast cancer. Oncotarget 2017;8:38294-308.

42. Wang X, Ji X, Chen J, Yan D, Zhang Z, Wang Q, Xi X, Feng Y. SOX2 enhances the migration and invasion of ovarian cancer cells via Src kinase. PloS one 2014;9:e99594.

43. Liu F, Cao J, Wu J, Sullivan K, Shen J, Ryu B, Xu Z, Wei W, Cui R. Stat3-targeted therapies overcome the acquired resistance to vemurafenib in melanomas. The Journal of investigative dermatology 2013;133:2041-9.

44. Lee TK, Castilho A, Cheung VC, Tang KH, Ma S, Ng IO. CD24(+) liver tumor-initiating cells drive self-renewal and tumor initiation through STAT3-mediated NANOG regulation. Cell stem cell 2011;9:50-63.

45. Zhao C, Li H, Lin HJ, Yang S, Lin J, Liang G. Feedback Activation of STAT3 as a Cancer Drug-Resistance Mechanism. Trends in pharmacological sciences 2016;37:47-61.

46. Wang W, Wang X, Peng L, Deng Q, Liang Y, Qing H, Jiang B. CD24-dependent MAPK pathway activation is required for colorectal cancer cell proliferation. Cancer science 2010;101:112-9.

47. Niu G, Bowman T, Huang M, Shivers S, Reintgen D, Daud A, Chang A, Kraker A, Jove R, Yu H. Roles of activated Src and Stat3 signaling in melanoma tumor cell growth. Oncogene 2002;21:7001-10.

48. Wang X, Zhang Y, Zhao Y, Liang Y, Xiang C, Zhou H, Zhang H, Zhang Q, Qing H, Jiang B, Xiong H, Peng L. CD24 promoted cancer cell angiogenesis via Hsp90-mediated STAT3/VEGF signaling pathway in colorectal cancer. Oncotarget 2016;7:55663-76.

49. Ohanna M, Cheli Y, Bonet C, Bonazzi VF, Allegra M, Giuliano S, Bille K, Bahadoran P, Giacchero D, Lacour JP, Boyle GM, Hayward NF, et al. Secretome from senescent melanoma engages the STAT3 pathway to favor reprogramming of naive melanoma towards a tumor-initiating cell phenotype. Oncotarget 2013;4:2212-24.

50. Salnikov AV, Bretz NP, Perne C, Hazin J, Keller S, Fogel M, Herr I, Schlange T, Moldenhauer G, Altevogt P. Antibody targeting of CD24 efficiently retards growth and influences cytokine milieu in experimental carcinomas. British journal of cancer 2013;108:1449-59.

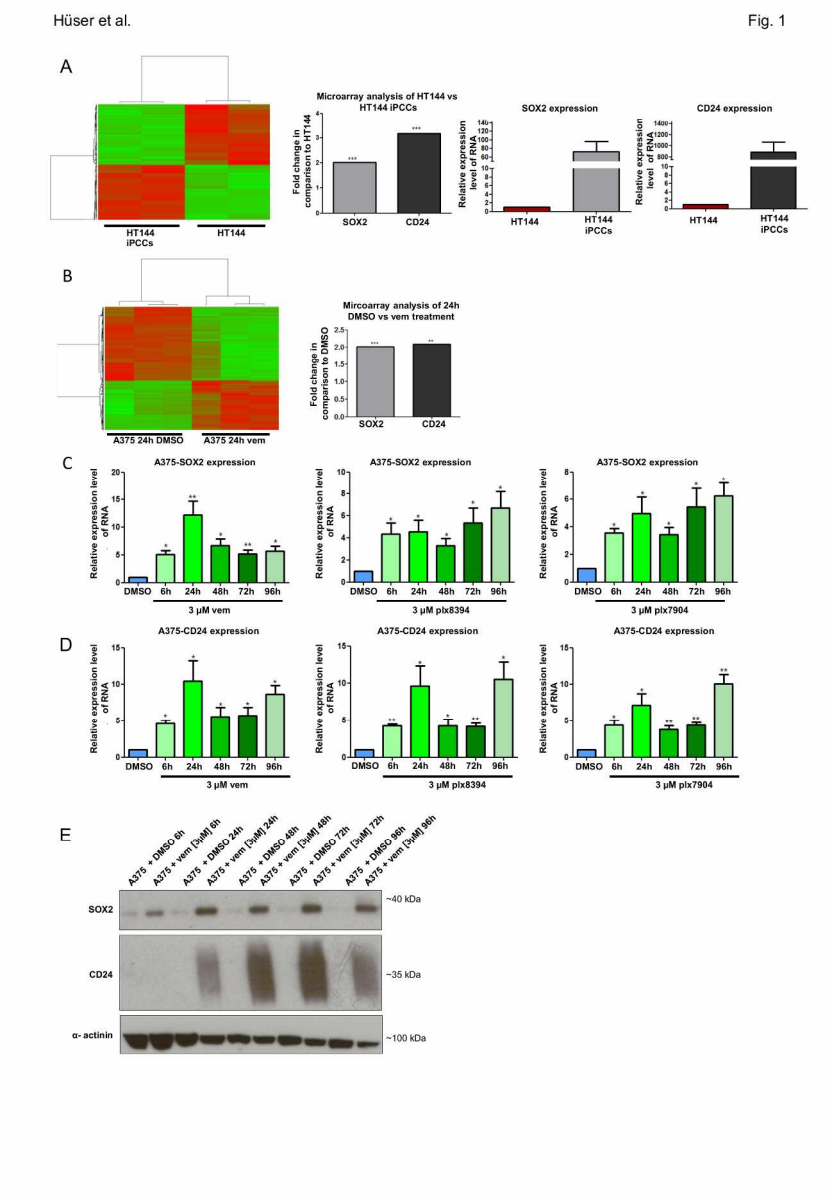


Figure 1: SOX2 and CD24 are highly upregulated in BRAFi resistant melanoma iPCCs and in short-time BRAFi treated melanoma cells

A: Microarray data from iPC-HT144 cells in comparison to the parental HT144 cells show a significant upregulation of SOX2 and CD24 in the iPCCs (fold change in log2 scale). Next 2 graphs show validation of microarray data by qPCR. qPCR data are shown as the relative expression level of mRNA, normalized to 18S (n=2). B: Microarray data from A375 treated for 24h with 3 µM vem or the corresponding amount of DMSO show significant upregulation of SOX2 and CD24 after vem treatment. C: SOX2 is significantly upregulated after different time periods of BRAFi (vem, plx8394, plx7904) treatment [3 µM] in A375 cells. D: CD24 is significantly unregulated after treatment with BRAFi in A375 cells. E: The Expression of SOX2 and CD24 under vem treatment was analysed by western blot at different time points as indicated. SOX2 and CD24 expression is highly increased after vem treatment. ($p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***)

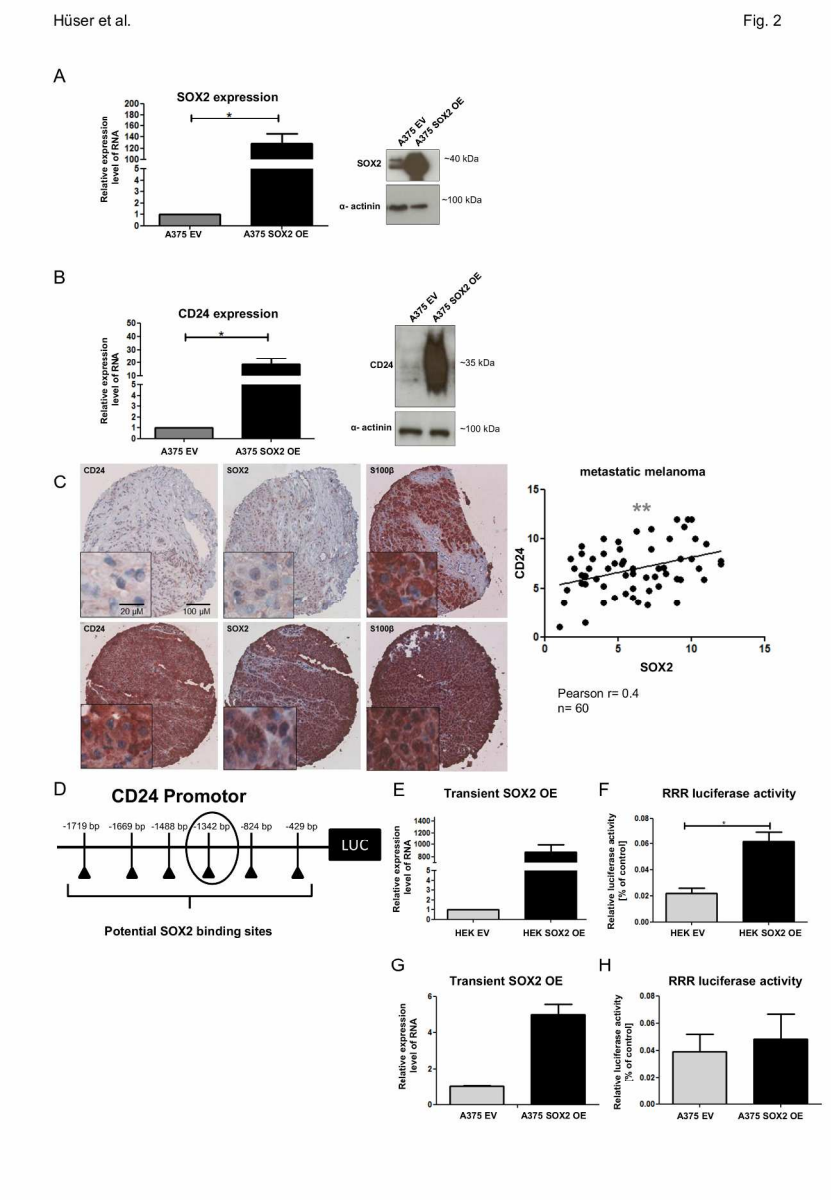
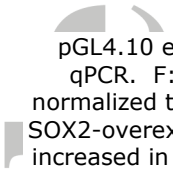


Figure 2: CD24 is regulated by SOX2 and their levels are correlated in metastatic melanoma patient samples A: Validation of SOX2 overexpression (OE) in A375 cells by qPCR showing relative expression level of mRNA, normalized to 18S and on protein level in WB. EV= empty vector B: qPCR and WB demonstrating an increased expression of CD24 in A375 SOX2 OE cells. C: Left: IHC staining of a TMA of metastatic melanoma stained for SOX2, CD24 and S100β as indicated. The inlay shows the same sample at a higher magnification. The top row shows an example for a low score of SOX2 and CD24 while the sample for sure contains tumor cells as demonstrated by S100β. The lower row shows a TMA sample highly positive for SOX2 and CD24 while S100β demonstrate the presents of tumor cells. Right: Pearson's correlation analysis of CD24 and SOX2 IHC reveal positive correlation of SOX2 and CD24 expression levels in metastatic melanoma samples of patients. D: Schematic drawing of CD24 promotor with its potential SOX2 binding sites followed by the luciferase reporter as it is used in the luciferase assay. The most likely binding site due to ChIPseq data is circled. The plasmids were transiently transfected into HEK and A375 cells either together with a SOX2 OE plasmid or an EV control for 48 h. The CMV promotor served as positive control and the



pGL4.10 empty vector was used as negative control. E: Transient OE of SOX2 in HEK cells validated by qPCR. F: The luciferase activity dependent on the SOX2 expression level was measured and all were normalized to the Renilla luciferase activity. The Relative Response Ratio (RRR) is significantly higher in the SOX2-overexpressing cells. G: Transient SOX2 OE in A375 cells as validated by qPCR. H: The RRR is slightly increased in A375 cells overexpressing SOX2 in comparison to EV control cells. ($p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***)

190x275mm (300 x 300 DPI)

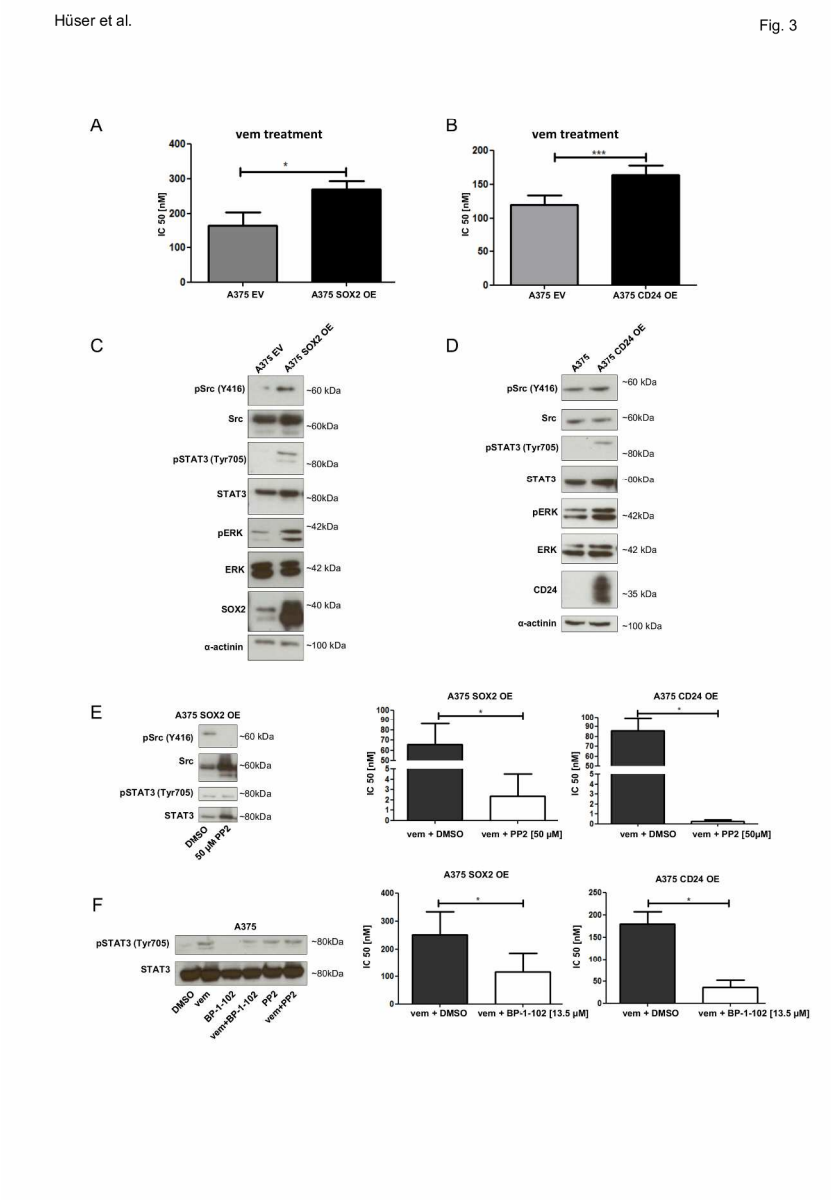


Figure 3: Upregulation of SOX2 or CD24 goes along with increased resistance towards BRAFi and activation of Src, STAT3 and ERK

A: A375 SOX2 OE cells show a higher IC50 than empty vector (EV) control cells and are therefore less sensitive towards vem. B: A375 CD24 OE cells show a higher IC50 when compared to EV control cells. Therefore these cells can tolerate a higher concentration of vem C: WB showing an increased activation of Src, STAT3, and ERK in SOX2-overexpressing A375 cells. D: CD24 overexpression in A375 results in an increased activation of Src, STAT3 and ERK. E: A375 SOX2 OE cells were stimulated with PP2 [50 µM], an inhibitor of Src. WB showing Src inactivation but no changes in STAT3 activation. Treatment of A375 SOX2 OE or A375 CD24 OE cells with PP2 [50 µM] in addition to vem leads to a decreased IC50. F: Left: STAT3 is activated by vem [3 µM] and can be inhibited by BP-1-102 [15 µM] but not by PP2 [50 µM]. Right: the more resistant A375 SOX2 OE/ CD24 OE cells can still be killed by the STAT3 inhibitor BP-1-102 as demostrated by the lower IC50 value of the in addition to vem with BP-1-102 treated cells. ($p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***)

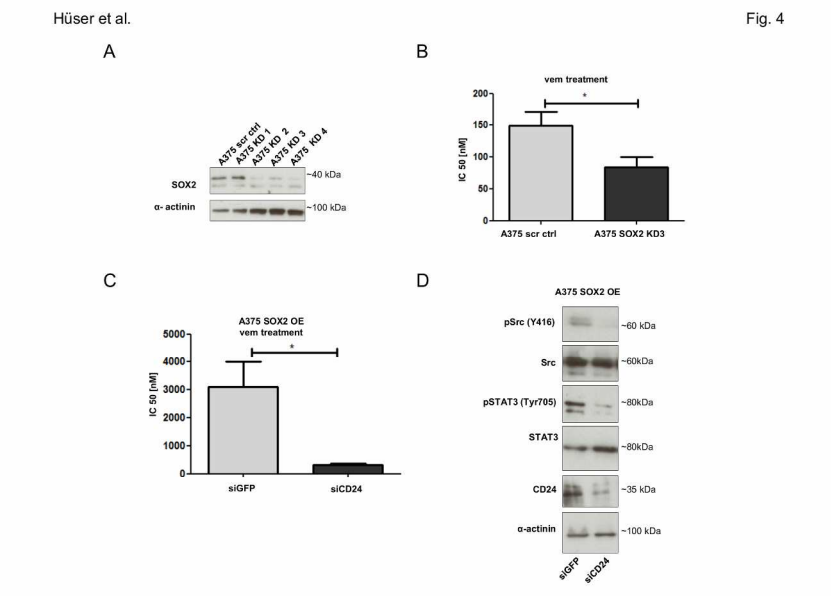


Figure 4: KD of SOX2 or CD24 causes a higher sensitivity towards BRAFi treatment
A: WB demonstrating that the shRNA-mediated KD of SOX2 in A375 cells is effective. B: A375 cells with a KD of SOX2 show a higher sensitivity towards vem. C: siRNA KD of CD24 in A375 SOX2 OE cells results in a decreased resistance towards vem as shown by the lower IC50 in the CD24 KD cells. D: siRNA KD of CD24 leads to a decreased activation of Src and STAT3 in A375 SOX2 OE cells. ($p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***)

190x275mm (300 x 300 DPI)

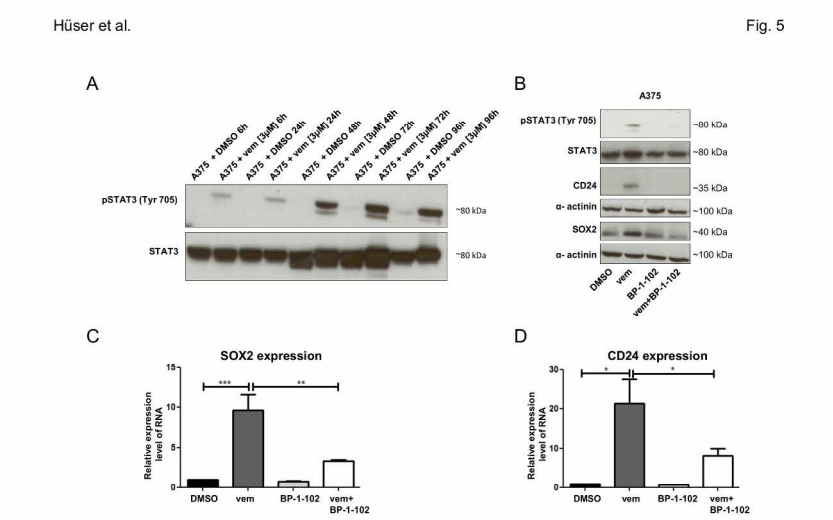


Figure 5: STAT3 activation by vem is essential for increased SOX2 and CD24 expression
A: WB of A375 treated for the indicated times with 3 µM vem or DMSO as control. The STAT3 activation is depicted by pSTAT as well as the total STAT3 level as detected by WB as shown. B: WB of A375 treated for 24h with DMSO, vem [3 µM], BP-1-102 [15 µM], vem [3 µM] + BP-1-102 [15 µM]. The WB demonstrats the increased STAT3 activation upon vem treatment can be inhibited in the presence of the STAT3 inhibitor BP-1-102. Furthermore, the vem induced SOX2 and CD24 expression is reduced if the STAT3 inhibitor is present in addition to vem. C/D: qPCR data which confirming the WB results for SOX2 and CD24 on the mRNA level. qPCR showing relative expression level of mRNA, normalized to 18S. ($p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***)

190x275mm (300 x 300 DPI)

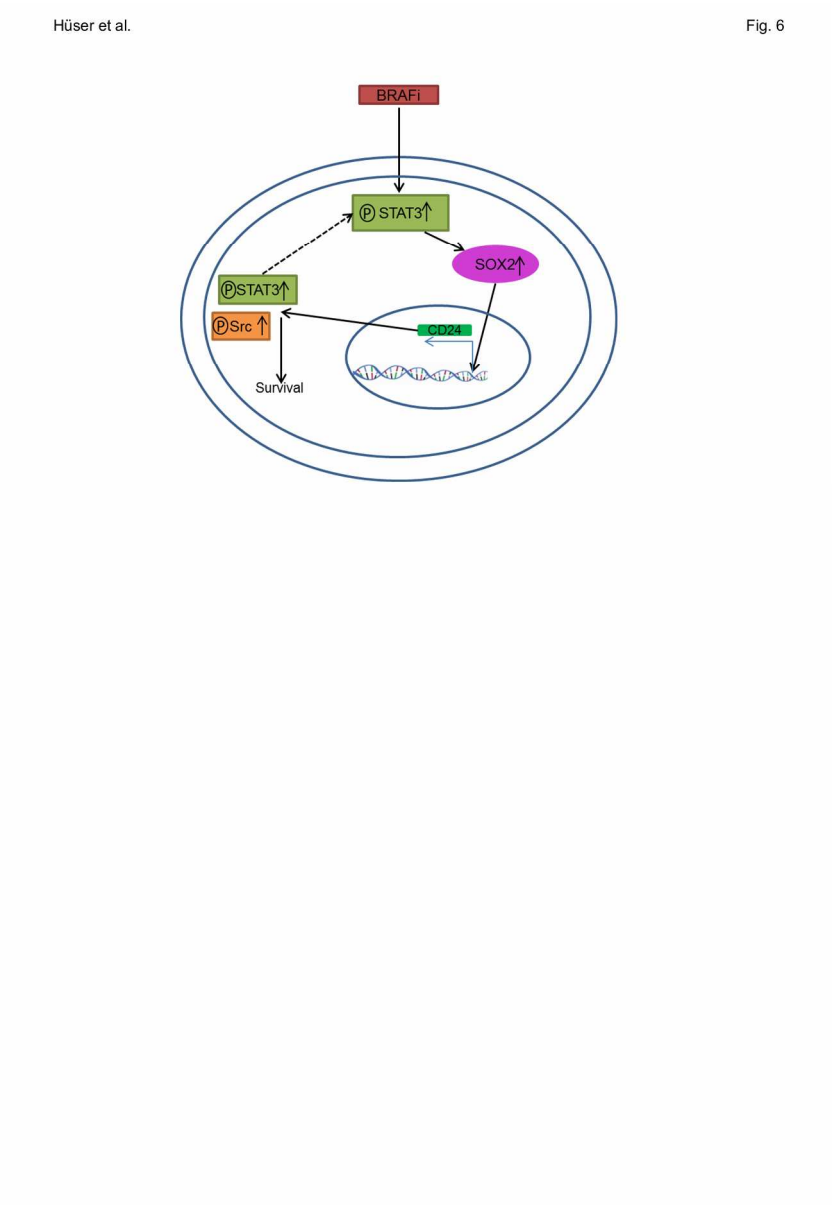


Figure 6: Schematic overview of the SOX2 and CD24-mediated adaptive resistance
The treatment with BRAFi leads to an activation of STAT3 that induces a higher expression of SOX2. SOX2 promotes the expression of CD24 by binding to its promotor. The increased CD24 expression causes a higher Src and STAT3 activation contributing to cell survival. STAT3 may act as an autocrine stimulus for further SOX2 expression.

190x275mm (300 x 300 DPI)