Dissertation

A Model Integrating Acquired Drug Resistance in Cancer

Submitted By

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Declaration

I hereby declare that this dissertation is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this dissertation. Due acknowledgement has been made in the text to all other material used. Throughout this dissertation and in all related publications I followed the guidelines of Good Scientific Practice.

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I would like to extend my gratitude to the PhD program `Molecular Medicine of the Medical University of Graz and the FWF for their generous funding which made all of this possible.

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CONTENTS

ABBREVIATIONS

ABCB5	:	ATP-Binding Cassette, Sub-Family B (MDR/TAP), Member 5
BCL-2	:	B cell lymphoma 2
BRAF	:	V-raf murine sarcoma viral oncogene homolog B1
CD133	:	Prominin1
CD271	:	Nerve Growth Factor Receptor
CD34	:	Hematopoietic Progenitor Cell Antigen CD34
CD38	:	Cyclic ADP-Ribose Hydrolase
CD44	:	CD44 molecule (Indian blood group)
CRAF	:	RAF proto-oncogene serine/threonine-protein kinase.
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
EMTR	:	Epithelial to mesenchymal transition regulator
ERK	:	Extracellular signal-regulated kinase
GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase
H3K27me3	:	Trimethyl Histone 3 lysine 27
H3K4me3	:	Trimethyl Histone 3 lysine 4
H3K9me3	:	Trimethyl Histone 3 lysine 9
HDAC	:	histone deacetylase
HGF	:	Hepatocyte growth Factor
IDTC	:	Induced drug tolerant cells
IL-2	:	Interleukin 2
MAP3K8	:	Mitogen-Activated Protein Kinase Kinase Kinase 8

MEK	:	Mitogen activated protein kinase
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-KappaB	:	Nuclear factor kappa-light-chain-enhancer of activated B cells
NRAS	:	Neuroblastoma RAS viral (v-ras) oncogene homolog
P21	:	cyclin-dependent kinase inhibitor 1
P27	:	P27 kip1 protein
PDGFR β	:	Platelet-derived growth factor receptor beta
SCID	:	severe combined immunodeficiency
sh-RNA	:	Short hairpin Ribonucleic acid
Slug	:	Zinc finger protein SNAI2
SMAD4	:	SMAD Family Member 4
Snail	:	Zinc finger protein SNAI1
Stat3	:	Signal transducer and activator transcription factor3
TAM	:	Tumour associated macrophages
TGF-β1	:	Transforming Growth factor Beta 1
TNF α RI	:	Tumour necrosis factor alpha receptor 1
TNF α	:	Tumour Necrosis factor alpha
Twist	:	Twist-related protein 1
Tyr	:	Tyrosine

SUMMARY

Acquired drug resistance is a major challenge for the successful treatment of any cancer. Even with the advent of molecular targeted therapy adding to the already existing therapies the occurrence of resistance still prevailed. The key and obvious primary reason for the phenomenon of acquired drug resistance is that the cells are surviving the initial hit by the drug. Various reasons have been incriminated to allow for the survival of residual cells after treatments. These include extrinsic factors like growth factors and cytokines in the tumour microenvironment or which are secreted by the cancer cells themselves. Intrinsic factors also have been attributed to be important which include the activation of alternative pathways seen to be a natural response to inhibition or targeting of cancer driving genes. Another aspect of intrinsic factors responsible for acquired drug resistance is the presence of multiple drug tolerant stem like cells which are reported to be enriched in the residual population. So far the existing literature has delineated divergent mechanisms contributing to multiple drug resistance in this population including drug efflux mechanisms and chromatin modifications. However, the underlying cause for the occurrence of a multiple drug resistant cancer stem cell like population has been quite obscure as the only explanation that has been given so far is that they pre-exist in the parent population. It also needs to be determined whether there is a link between intrinsic and extrinsic factors. Hence this study aimed at tackling these questions using a BRAF mutant melanoma model for which BRAF and MEK inhibitors have currently been developed as a primary treatment strategy. In the course of these studies the response of melanoma cells to BRAF and MEK inhibitors was observed to be largely affected by the presence of growth factors and cytokines like TGF-β1 and TNF-α. The regulation of Twist1, an anti-apoptotic protein, through these pathways seems to determine the number of residual cells surviving the drug exposure.

Cells surviving after 12 days of drug exposure were observed to be always multiple drug tolerant irrespective of the condition and drug they were exposed to. In an effort to characterize the reasons behind that I observed that cancer cells have a time dependent general intrinsic capability to exhibit an innate primary response to adverse environmental conditions like hypoxia or low glucose, but which is also triggered by drug exposure. This response induces a multiple drug tolerant stem like state in the cancer cells which drives the process of acquired drug resistance. The

multiple drug tolerant state was characterised by the induction of stem cell markers like CD271, ABCB5 and increased ALDH activity along with global chromatin remodelling. Moreover the cells at that state exert a higher angiogenic potential making the population highly tumorigenic. These multiple drug tolerant cells are also characterized by the activation of multiple signalling cascades that contribute to highly active AKT and ERK survival pathways making them even more difficult to be targeted. So it seems that the micro-environmental conditions that exist at the time of exposure to drugs, including the presence of growth factors and cytokines, determine which percentage of cells might survive the initial drug exposure exhibiting an innate adaptive response to gain multiple drug tolerant properties. Together my studies point to a unifying model of acquired drug resistance, which could be used to significantly improve the administration of anticancer treatment regimens eventually leading to a sustainable disease.

ZUSAMMENFASSUNG

Einer der größten Hindernisse für eine erfolgreiche Krebstherapie ist die Therapieresistenz. Auch moderne Therapieformen wie die zielgerichtete molekulare Therapie stellen hier keine Ausnahme dar. Für den Erfolg der Therapie ist dabei die Tatsache, dass jene Krebszellen, die die anfängliche Therapie überleben dann resistent werden von ausschlaggebender Bedeutung. Die Ursachen für die Medikamentenresistenz sind mannigfach, hervorzuheben sind jedoch zwei prinzipielle Überlegungen, die auch schon experimentell gut untermauert sind. Sowohl äußerliche als auch innerliche Faktoren tragen zur Resistenzentwicklung bei. Äußerliche wie die Präsenz von Wachstumsfaktoren und Zytokinen im Tumormilieu und innerliche wie das Aktivieren von alternativen Signalwegen und stammzellähnlichen Mustern. All diese Veränderungen bewirken in unterschiedlichem Ausmaß, dass die Krebszelle eine Resistenz gegenüber dem Medikament entwickelt. Welche Krebszellen nun dafür prädestiniert sind besonders schnell und wirkungsvoll resistent zu werden ist nur unvollständig bekannt. Bisher wurde angenommen, dass nur eine geringe Anzahl der Zellpopulation insgesamt diese Fähigkeit hat. Um dieser Frage nachzugehen und einen möglichen Zusammenhang zwischen äußerlichen und inneren Faktoren herzustellen wurden BRAF mutierte Melanomzellen untersucht, da BRAF und MEK Inhibitoren zur Behandlung von Melanompatienten im fortgeschrittenen Stadium verwendet werden und die sich daraus ergebende Resistenzentwicklung gut dokumentiert ist. Im Zuge dieser Untersuchungen konnte ich nun nachweisen, dass zwei Zytokine, die im ausreichenden Maße im Tumormilieu vorhanden sind, nämlich TGF-□1 und TNF-□ in unterschiedlicher Weise die Resistenz von Melanomzellen beeinflussen. Darüberhinaus steuern beide die Expression eines antiapoptotischen Transkriptionsfaktors, Twist1, der wiederum eine Vielzahl von Genen reguliert, die zur Resistenz beitragen.

Jene Melanomzellen, die über einen Zeitraum von zwölf Tagen einem Medikament ausgesetzt waren und überlebten waren überraschenderweise gegenüber einer großen Anzahl an Medikamenten tolerant. Diese Zellen, die einen eigenen spezifischen Phänotyp darstellen, wurden als induzierte medikamententolerante Zellen (IMTZ) bezeichnet, die eine generische Form der Resistenz aufweisen. IMTZ entwickelten sich auch unter unwirtlichen Bedingungen, wie zum Beispiel Hypoxie

oder einem niedrigen Glukosegehalt im Medium. Interessanterweise exprimieren IMTZs Stammzellmarker wie CD271, ABCB5 und zeigen eine erhöhte ALDH Aktivität. Desweiteren beobachtete ich eine erhöhte Tumorigenität im Mausmodell verbunden mit einem ebenfalls erhöhten angiogenetischen Potential, das zu einer höheren Anzahl an Blutgefäßen führte und Veränderungen im Chromatinmuster. Im Speziellen kam es zu einer vermehrten Aktivierung des AKT und ERK Signalweges, die ebenfalls zur Resistenz beitrug. IMTZ entwickeln sich zeit- und konzentrationsabhängig sowie abhängig von äußeren Faktoren, die bestimmen, wie viele der Krebszellen überleben und resistent werden. Auf Basis der IMTZ wurde ein stadienabhängiges Modell entworfen, das die Entwicklung der erworbenen Resistenz als zeitliches Phänomen beschreibt, dem die Resistenz in Form einer Toleranz gegenüber vielen Medikamenten, als multiple Medikamentenresistenz vorangeht. Die weitere Erforschung dieser Resistenzform ist wichtig um Strategien zu entwickeln, wie diese umgangen oder zumindest reduziert werden kann.

CANCER DRUG RESISTANCE AN OVERWIEW

With the recent advances in cancer research there has been a tremendous increase in possible cancer treatment strategies and an expansion of anti-cancer drugs for various types of cancers. The mode of treatment has also turned from generic chemotherapeutic treatment strategies to specific targeted therapies in the form of small molecular inhibitors and antibodies (Maemondo et al., 2010; Merelli et al., 2013; Tuveson et al., 2003) which has significantly increased the response rates in multiple cancer types. This being said, acquired drug resistance remains a major concern in multiple forms of cancer irrespective of the treatment strategy (Emery et al., 2009; Frank et al., 2005; Gowrishankar et al.; Li et al., 2009; Ma et al., 2008; Straussman et al., 2012; Ware et al., 2013; Wilson et al., 2012). The cause of acquired drug resistance has been attributed to both intrinsic factors like activation of survival pathways, mutation and epigenetic modifications(Cohen-Solal et al., 2011; Emery et al., 2009; Engelman et al., 2007; Longley and Johnston, 2005) as well as extrinsic factors that are present in the tumour micro environment including growth factors and cytokines (Wilson et al., 2012) (Figure 1).

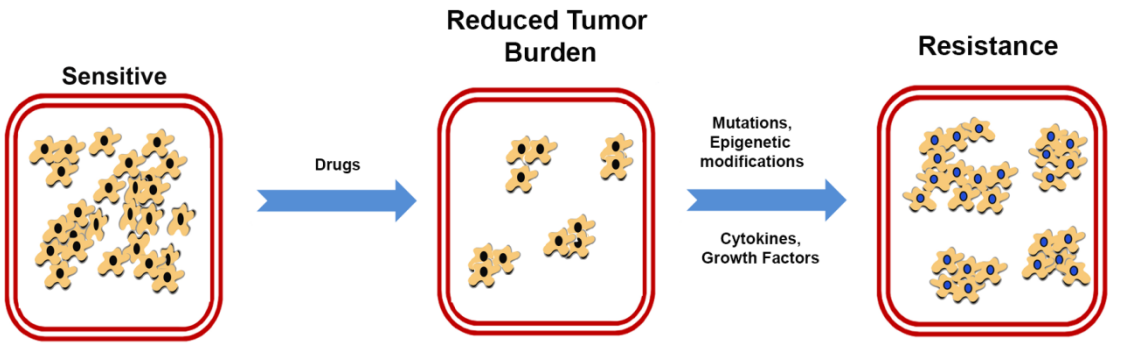

Figure 1: ***Emergence of Cancer drug resistance from reminiscent cells***: *Therapies usually induce a reduction in tumour burden but the surviving cells gain epigenetic modification or mutations with the support from tumour microenvironment to establish resistance.*

Melanomas are no exception to this phenomenon whereupon recent targeted small inhibitor molecules against mutated BRAF (Vemurafenib) and MEK (Trametinib) showed tremendous promise, while acquired drug resistance still was observed in most treated patients. Hence initially I started my research by focusing on drug resistance in melanoma against BRAF and MEK inhibitors.

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CHAPTER 1

Role of Twist1 in Melanoma Drug Resistance to BRAF and MEK Inhibitors

(1.1) Introduction

(1.1.1) Targeting BRAF mutation in melanomas

50-60% Melanomas (Figure 2A,B) carry a constitutively active BRAF through various mutations in its kinase domain which is mutually exclusive to NRAS mutations which are found in another 15% of melanoma patients (Brose et al., 2002; Tuveson et al., 2003). BRAF mutations in the kinase domain of the protein lead to a constitutively active MAPK –ERK signaling which lead to growth and survival of the cancer cells. This has encouraged pharmaceutical companies like Roche to develop inhibitors that specifically target mutated BRAF and MEK1,2 inhibitors. Vemurafenib (PLX4032) the latest of this kind had shown some promising results with almost 81% of patients initially responding to the therapy (Flaherty et al., 2010). In this regard the previous two FDA approved therapies high-dose IL-2 and dacarbazine are each associated with only a response rate of 10 to 20% (Atkins et al., 2000; Atkins et al., 1999; Flaherty et al., 2010). Recent studies with MEK inhibitors in BRAF mutant melanoma also showed very similar results (Flaherty et al., 2012).

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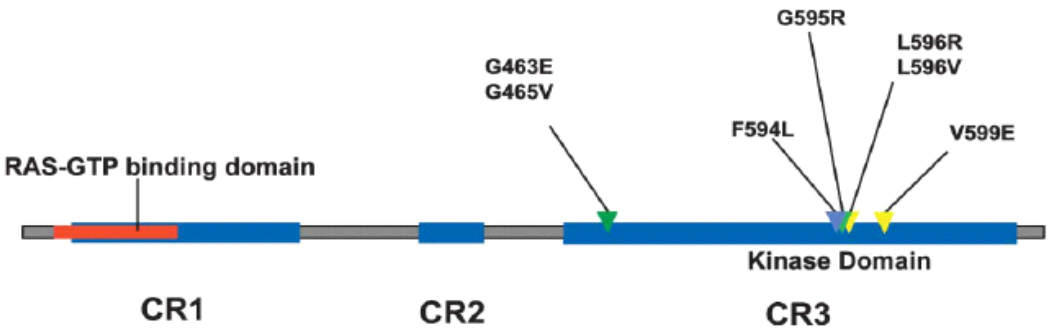
A)



B

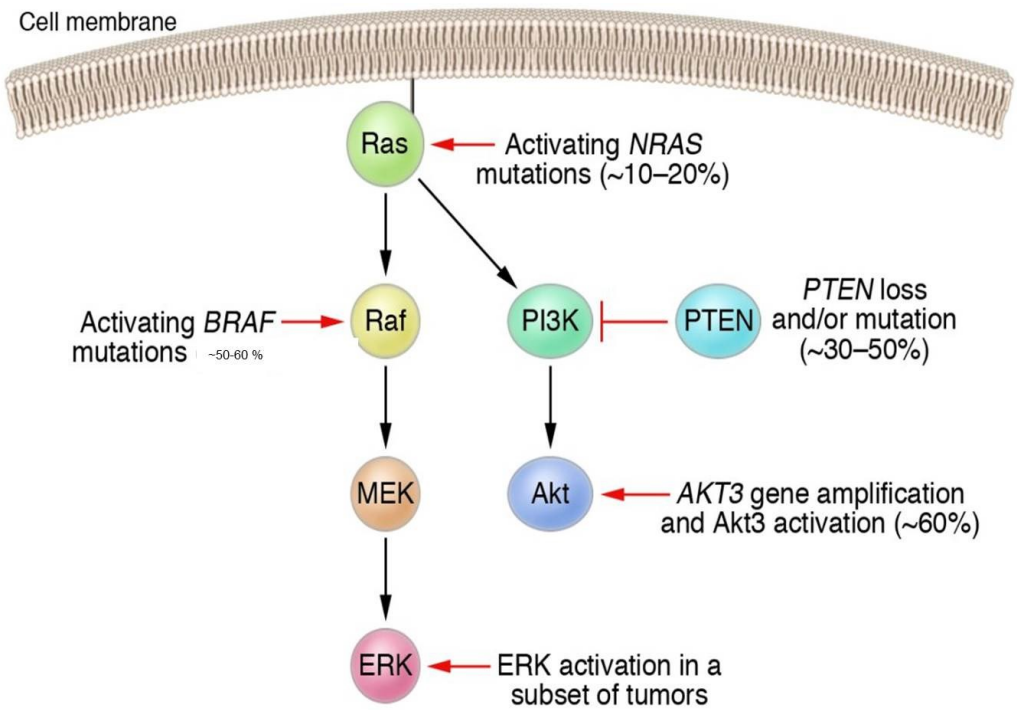


Figure 2*:*TARGETING CONSTITUTIVELY ACTIVE BRAF*:*A) Reported Mutations in the Kinase domain leading to constitutively active BRAF, Adopted from(Tuveson et al., 2003).B) Mutational dynamics of melanoma Adopted from (Chudnovsky et al., 2005)

But the ever repeating problem with these therapies in common with the pre-existing therapies is the resurgence of cancer due to acquired resistance. The emergence of acquired drug resistance has been reported to be through up regulation of other factors or secondary mutations and also the lack of complete responses, which all looks to be contributing to the problem. The number of patients experiencing a complete response in PLX4032 clinical trials was just 2-3% out of the initial 81% response (Flaherty et al., 2010). Even this differential response was well observed by different groups *in vitro* where they saw some BRAF mutant cell lines responding not at all to the PLX4032 and some responding partially (Sondergaard et al., 2010). In accordance with this observation there was a report of relapse in the majority of patients, no longer being responsive to PLX4032. This has led to serious efforts being made to identify the reason for the acquired resistance in these cancer patients which was reported to be driven by multiple pathways, either through acquired mutations in NRAS (Nazarian et al., 2010), CRAF (Johannessen et al., 2010; Montagut et al., 2008) genes or through up regulation of PDGFR β (Nazarian

et al., 2010) or MAP3K8 (Johannessen et al., 2010). What is also interesting about these observations is that the NRAS mutation looks to be acquired during the process of treatment and was not a result of already pre-existing clones of cancer cells with NRAS mutation, as these cells were harbouring both BRAF and NRAS mutations which so far was reported to be mutually exclusive. The same was the case for the upregulation of PDGFR β which was not found in the primary lesions. (Nazarian et al., 2010). These studies strongly suggest that incomplete responses in patients would lead to partial eradication of parent tumours which on the course of treatment gain mutations or epigenetic changes leading to the establishment of drug resistant tumours.

(1.1.2) The role of antiapoptotic protein Twist1 in cancer drug resistance

Twist1 is a basic helix loop helix protein which has been reported to be a major epithelial to mesenchymal transition regulator (EMTR) (Ansieau et al., 2008). Epithelial to mesenchymal transition (EMT) is widely reported to be closely associated with drug resistance(Singh and Settleman, 2010) . Twist1 expression is also widely reported in various cancer types and multiple reports (Ansieau et al., 2008) also indicts its role as an anti-apoptotic protein. Hence it is not surprising that the role of Twist1 in drug resistance is widely reported (Kwok et al., 2005; Li et al., 2009; Shiota et al., 2013; Shiota et al., 2010).Also importantly Twist1 expression has been reported to induce an invasive and metastatic potential in cancer cells(Eckert et al., 2011; Weiss et al., 2012a; Yang et al., 2004).

Twist1 expression in cancer has been shown to be regulated by multiple signalling pathways including ERK 1,2 (Weiss et al., 2012a), NF-kappaB (Li et al., 2012) and AKT(Hong et al., 2009). Accordingly Twist1 expression has also been reported to be induced by multiple growth factors and cytokines.(Li et al., 2012; Shiota et al., 2012). In a previous report from our group we had observed the same in Melanoma cells where hepatocyte growth factor (HGF) was observed to regulate Twist1 expression through ERK, AKT and NF-kappaB signalling (Koefinger et al., 2011).But the regulation of Twist1 in BRAF mutant melanoma cell lines was observed to be more prominent through ERK signalling than through other pathways, which also explains their sensitivity to BRAF and MEK inhibitor. Recently HGF has been shown to be

involved in resistance to the BRAF inhibitor Zelboraf®, a finding, which underscores the importance of cytokines and growth factors found in the tumour environment and secreted by cancer cells alike contributing to drug resistance (Straussman et al., 2012; Wilson et al., 2012). Hence we predicted that regulation of Twist1 through multiple growth factors in the tumour microenvironment could determine drug resistance exhibited by BRAF mutant melanoma cells to the BRAF inhibitor PLX4032 and the MEK inhibitor GSK1120212.

(1.2)Results and Discussion

(1.2.1)TGF-β1 regulates Twist1 expression through Stat3, enhancing the response to BRAF/MEK inhibitors in melanoma

To identify potential cytokines and growth factors contributing to loss of sensitivity towards BRAF or MEK inhibitors, the effect of several growth factors in this context was tested. During this process I observed that TGF-β1 significantly affected the response of melanoma cells to both BRAF and MEK inhibitors. TGF-β1 is a highly potent growth factor which plays enormous and divergent roles including cell cycle progression, apoptosis, immune surveillance and differentiation (Reed et al., 1994). In the context of cancer TGF-β1 can be described as a double edged sword as it has been reported to contribute to tumour suppressive and pro oncogenic functions. For example loss of SMAD 4, a central mediator of TGF -β1 signalling and TGF beta signalling receptor 2 inactivating mutations is widely reported to be contributing to cancer progression in pancreatic cancer, colorectal cancer, and head and neck cancer. Increased expression of TGF-β1 is also associated with progress of colorectal cancer, prostate cancer, breast cancer and melanoma.

Treatment of WM164 cells with BRAF inhibitor PLX4032 (1μM) (Vemurafenib, Zelboraf®) or MEK inhibitor GSK1120212 (50 nM) resulted in caspase 3 mediated apoptosis, while the same inhibitors in combination with 5ng/ml of TGF-β1 almost doubled the induction of apoptosis (Figure 3A). MTT assays also revealed a similar trend where WM164 cells treated in combination with TGF-β1 showed a very low growth rate (Figure 3B).

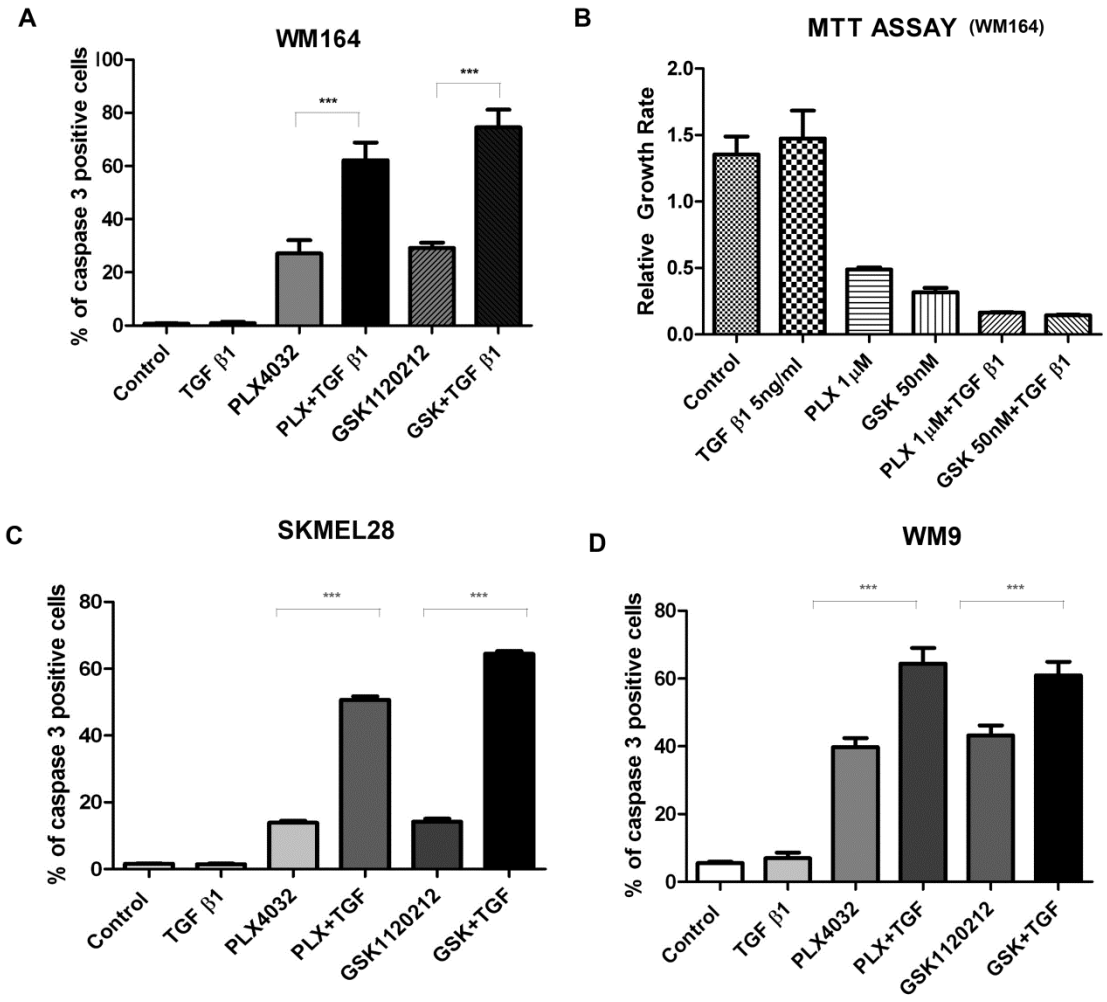


Figure 3: TGF-β1 ENHANCES PLX4032 AND GSK1120212 INDUCED APOPTOSIS: (A) *Percentage of cleaved caspase 3 positive cells after exposure to BRAF (PLX4032 1μM) and MEK inhibitors (GSK1120212 50nM) in combination with TGF-β1 (5ng/ml) or alone for 36 hrs in WM164. (B) MTT assays conducted in WM164 cells undergoing the treatment mentioned in (A).Percentage of cleaved caspase 3 positive cells after exposure to BRAF (PLX4032 1μM) and MEK inhibitors (GSK1120212 50nM) in combination with TGF-β1 (5ng/ml) or alone for 36 hrs, in SKMEL28(C) and WM9 (D). (***p-value <0.01- Student's t-test).(MTT assay performed by Ehsan Bonyadirad)*

The results were similar in BRAF mutant melanoma cell lines SKMEL28 and WM9 (Figure 3C, D) where increased caspase 3 mediated apoptosis was observed, while exposure to TGF-β1 alone did not induce any response. Correspondingly, inhibition of the TGF-β1 receptor with SB431542 partially rescued cell death induced by GSK1120212, suggesting that endogenous TGF-β1 produced by melanoma cells also contribute to the induction of apoptosis (Figure 4).

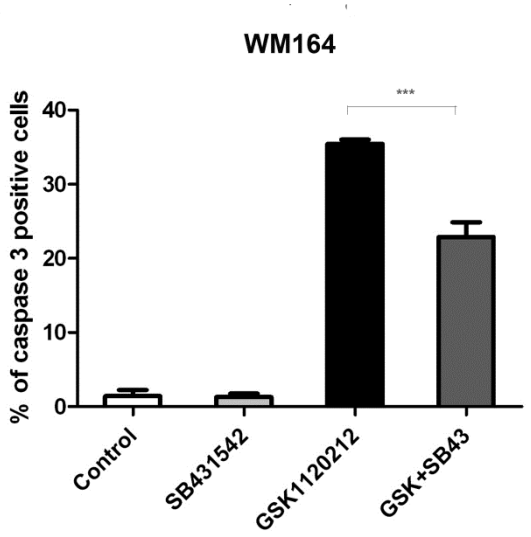


FIGURE4. ENDOGENOUS TGF-β1 PARTIALLY CONTRIBUTE TO GSK1120212 INDUCED APOPTOSIS: *Percentage of cleaved caspase 3 positive cells after exposure to MEK inhibitors (GSK1120212 50nM) in combination with SB431542 (TGF-*β1 inhibitor) for 36 hrs.

As mentioned earlier, the role of TGF-β1 in cancer progression can be described to be context dependent. Earlier studies have described TGF-β1 as a tumour suppressive factor as they could prevent the progression through cell cycles by induction of p15, p21 and p27(Elliott and Blobe, 2005), whereas studies have also described tumour promoting roles of TGF -β1(Maehara et al., 1999; Picon et al., 1998). The key to this differential regulation could be due to the primary response of cancer cells themselves to the growth factors and underlying signalling cascades. To understand this effect of TGF-β1 in enhancing apoptosis induction by PLX4032 and GSK1120212, further analyses of EMTRs like Slug, Snail and Twist1 were performed in each of this treatment conditions (Figure 5A,B). It was recognized that the inhibitor significantly affected expression of Twist1, whereas expression levels of other EMTRs like Slug and Snail did not correlate with the induction of apoptosis in WM164 cells. Combination of PLX4032 with TGF-β1 led to further down regulation of Twist1 expression, while treatment with TGF-β1 alone did not induce any significant difference.

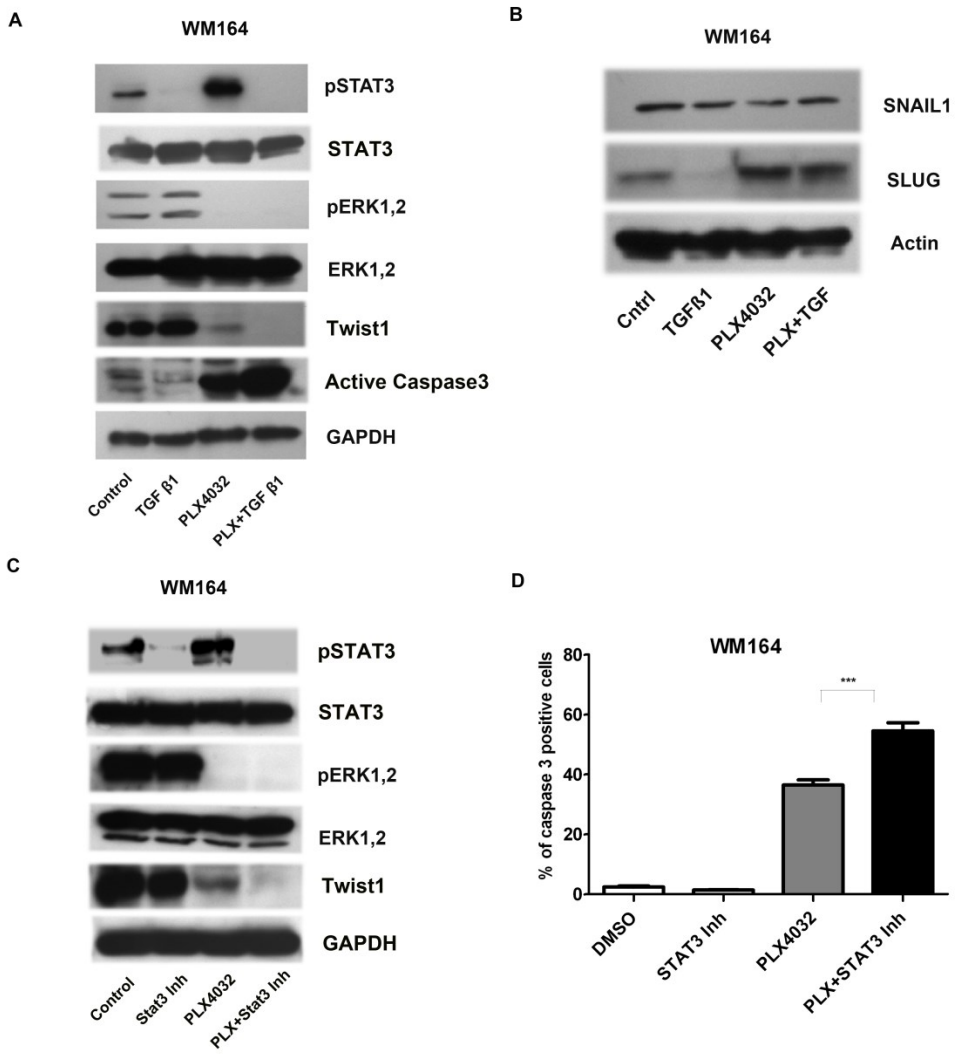


FIGURE 5. TGF-β1 REGULATES TWIST1 EXPRESIION THROUGH STAT3*:(A)Western blot analysis of total Stat3 and ERK, as well as phosphorylated Stat3 Tyr 705 (pSTAT3) along with ERK phosphorylation (pERK1,2), and its correlation with Twist1 down regulation and caspase 3 activation, in WM164 exposed to the BRAF inhibitor PLX4032 or TGF -β1 alone, or in combination of both. GAPDH is used as a loading control. (B) Western blot of Snail1 and Slug in WM164 cells treated cells with BRAF inhibitor or TGF-β1 alone, or in combination of both, with beta actin as a loading control.(C) Experiments mentioned in FIGURE 5 (A) repeated with PLX4032(1μM) in combination with Stat3 inhibitor (Stat3 inh VII (1 μM)) .(D) Percentage of cleaved caspase 3 positive cells after 36 hrs exposure to BRAF or Stat3 inhibitor (Stat3 inh VII (1 μM)) alone or in combination (***p-value <0.01- Student's t-test)*

Previous reports have suggested that STAT3 Tyr 705 phosphorylation can transcriptionally regulate Twist1 (Cheng et al., 2008). Activation of STAT3 Tyr 705 is also reported to be a natural feedback mechanism towards inhibition of ERK

signalling (Chung et al., 1997) and also leads to the nuclear translocation of STAT3 and transcriptional activation of downstream targets (Liu et al., 2008). Hence I analysed Stat3 phosphorylation along with ERK 1,2 under various treatment conditions and observed that the use of BRAF inhibitors as expected inhibited ERK1,2 phosphorylation which immediately led to the activation of STAT3 Tyr 705. Further combination of TGF-β1 with PLX4032 or alone was sufficient to inhibit, induced or endogenous STAT3 Tyr 705 phosphorylation (Figure 5A). This observation is in line with a previous report which has suggested the role of TGF-β1 in inhibiting STAT3 Tyr 705 phosphorylation (Wierenga et al., 2002).To confirm, whether the inhibition of STAT3 is contributing to the apoptosis enhancing effect of TGF-β1, I tested whether the combination of PLX4032 with STAT3 inhibitor (STAT3 inh VII) could reduce the Twist1 levels further in WM164 cells(Figure 5C). The effect was observed to be similar and also the combination led to a significant increase in apoptosis induction in WM164 cells (Figure 5D).

All together our results suggest that combinations of TGF -β1 with either BRAF or MEK inhibitors seem to enhance the apoptosis induction through inhibition of STAT3 activation. The role of Twist1 in this context required further testing. To test this I generated WM164 (WM164-T) and WM35 (WM35-T) BRAF mutant melanoma cells overexpressing Twist1 by stable lentiviral transduction and selection with G418. The overexpression of Twist1 in both of these cell lines was confirmed by immunoblotting (Figure 6A,B).

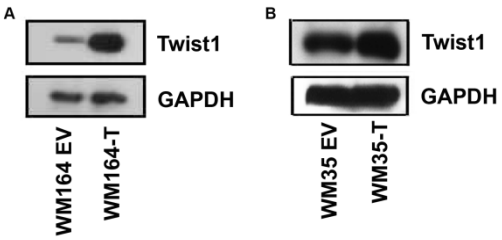


FIGURE 6:Twist1 viral transduction: *Immunoblots showing Twist1 overexpression in WM164 (WM164-T) (A)and WM35 (WM35-T)(B) when compared to WM164 (WM164 EV) and WM35 (WM35 EV) empty vector transduced cells.*

Further the cell lines were treated with PLX4032 or GSK1120212 in combination with TGF-β1 or alone to test their susceptibility. WM164-T and WM35-T cells showed significant resistance to both BRAF and MEK inhibitors and even to their combination with TGF-β1 (Figure 7A,B), suggesting that Twist1 contributes significantly to both BRAF and MEK inhibitor resistance.

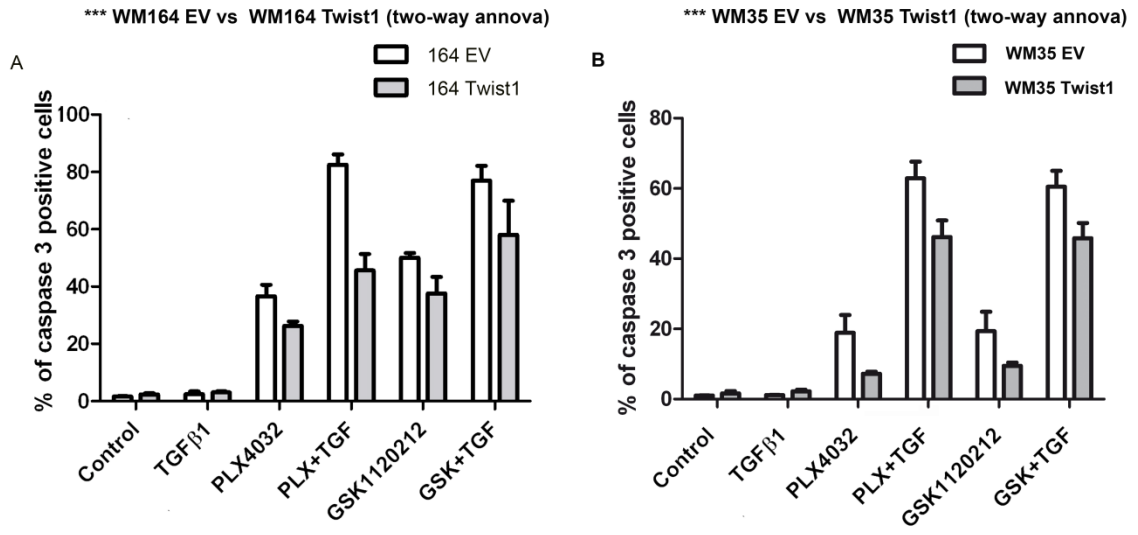


Figure 7: Twist1 induces resistance to BRAF and MEK inhibitors.(A)Twist1 overexpression in WM164 cells (164 Twist1) and WM35 (WM35 twist1) reduces the induction of apoptosis to treatment with PLX4032(1 μM) and GSK1120212 (50nM) even in combination with TGF-β1(5ng/ml) if compared to empty vector control (164 EV, WM35 EV) (***p-value <0.01- two-way ANOVA)

To further strengthen our finding WM164 Twist1 knockdown (WM164-sh-T) cells were generated using lentiviral vectors and selected with puromycin. The knockdown of Twist1 was confirmed by immunoblotting in comparison to the control sh RNAs transduced WM164 cells (WM164-sh-Cntrl). Further WM164-sh-Cntrl and WM164-sh-T were subjected to treatments with PLX4032 or GSk1120212 in combination with TGF -β1 whereupon Twist1 knockdown cells were observed to be more susceptible to the treatment. Also I further analysed the impact of knockdown or overexpression of Twist1 on long term survival against BRAF inhibitors by using WM164-T, WM164-sh-T and WM164 control cells. To test this, cells were plated in duplicates and treated for 12 continuous days with 1μM PLX4032. Media was changed every 3 days and another set of cells were kept in media containing equivalent volume of DMSO as used for dissolving PLX4032. After 12 days of exposure the cells were fixed and stained with crystal violet and representative pictures were taken .Further quantification was done by dissolving the dye from each well and the corresponding optical density of the dye from each well was measured.

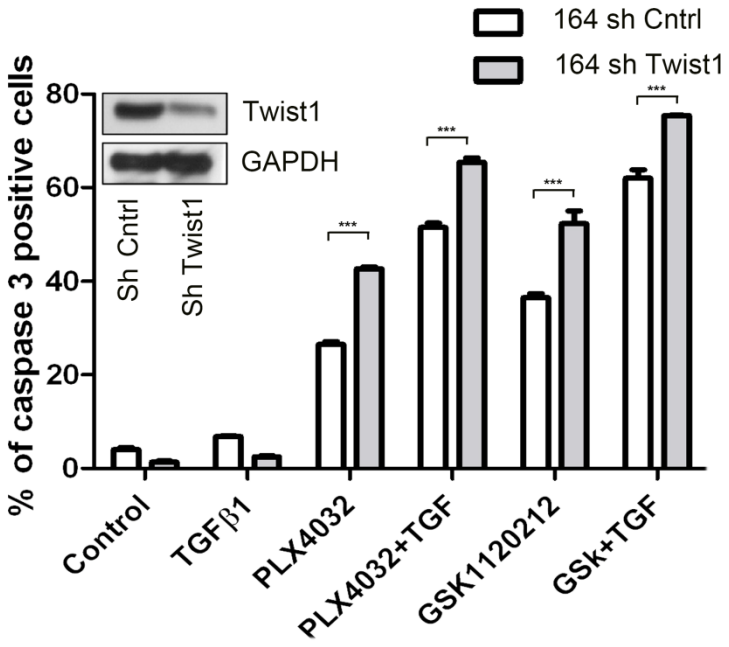


Figure 8: Twist1 Knockdown Enhances the response to BRAF and MEK inhibitors*.WM164 sh Twist1 transduced cells(164 sh Twist1) and Control sh RNA transduced cells were subjected to treatment with BRAF (PLX4032 1μM) and MEK inhibitors (GSK1120212 50nM) in combination with TGF-β1 (5ng/ml) or alone and cells were subjected to active caspase 3 analysis by facs*

The results clearly suggested a highly significant impact of Twist1 where Twist1 knockdown drastically reduced the number of surviving cells and Twist1 over expression significantly enhanced the survival of WM164 cells against BRAF inhibition.

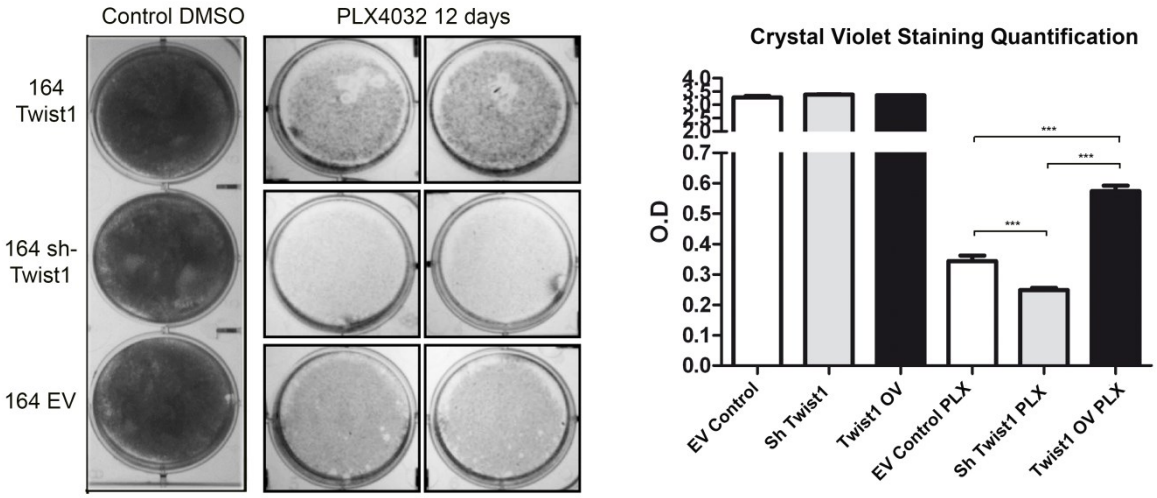


Figure 9: Twist1 Long term Survival Assay: *Crystal violet staining of WM164 empty vector control cells (164 EV), Twist1 overexpressing (164 Twist1) and Twist1 knockdown (164 sh Twist1) exposed to PLX4032 for 12 days (left). Quantification of the crystal violet staining was done at 590nm by a microtiter plate reader (right).*

Discussion

Together our results indicate that cytokines in the tumour microenvironment could affect Twist1 levels in BRAF mutant melanoma cells thereby affecting their response to BRAF and MEK inhibitors. Presence of TGF-β1 in the tumour microenvironment could be a major factor affecting the response of patients to BRAF and MEK inhibitors. The growth inhibitory effect of TGF-β1 in neoplasms, primary cells and cancer cells are observed in early phases and have been shown to be effective on epithelial like cells(Herman and Katzenellenbogen, 1994; Rodeck et al., 1994; Valverius et al., 1989), while cancer cells seem to loose their response to TGF-β1 as they progress(Parsons et al., 1995; Welch et al., 1990; Yan et al., 1992). Further cancer cells which are highly tumourigenic and aggressive are reported to constitutively secrete TGF-β1 (Arrick et al., 1992), which is also reported to induce invasive and metastatic properties. The same has also been reported in melanoma where an increase in TGF -β1 has been reported as the disease progresses and is associated with melanoma metastases(Krasagakis et al., 1998) (Reed et al., 1994). This could also be the reason why treatment with TGF-β1 itself did not induce any response in these BRAF mutant cancer cells. However the role of TGF-β1 in the context of BRAF and MEK inhibitors is significant as it down regulates Twist1 expression through inhibition of STAT3 phosphorylation which seems to be a natural response to BRAF or MEK inhibition. This natural feedback loop of STAT3 activation seems to ensure partial survival of the cancer cells in response to BRAF and MEK inhibitors. Hence a combination of STAT3 inhibition with either a BRAF or MEK inhibitor could be thought of as a strategic approach for treatment of BRAF mutant melanomas, which could improve the response in patients and would provide a longer disease free survival period.

(1.2.2) Role of TNF-alpha in inducing melanoma drug resistance to BRAF and MEK inhibitors through Twist1

From the previous results it became clear that the level of Twist1 in the cells could significantly affect the response of BRAF mutant melanoma cells to BRAF and MEK inhibitors. TGF-β1 seems to reduce the level of Twist1 in the context of BRAF or MEK inhibitors and hence to enhance the effect of inhibitors on the cells. Therefore it was rational to postulate that growth factors or cytokines that enhance the expression of Twist1 in the context of these inhibitors would lead to drug resistance. We had observed in the previous publication from our group, that HGF, which has also been reported to induce drug resistance against BRAF inhibitors (Straussman et al., 2012), induces Twist1 expression (Koefinger et al., 2011). Whereas Twist1 expression was induced by multiple pathways, the major driver of this expression was the MAPK-ERK pathway. Since the BRAF-ERK pathway is an activator of Twist1, which in this context will remain inhibited, I searched for growth factors that could possibly induce Twist1 expression independent of BRAF-ERK signalling.. TNF-α met these criteria as it is reported to induce Twist1 expression and epithelial to mesenchymal transition through the activation of NF-kappaB signalling in breast cancer (Li et al., 2012) and non-small cell lung cancer (Kumar et al., 2013). Twist1 up regulation through NF-kappaB has also been reported to induce resistance to chemotherapeutics in other cancer types by affecting BCL-2 signalling (Pham et al., 2007).

Similar to TGF-β1, the role of TNF α in cancer progression is not conclusive, as its function has been reported to be both tumor suppressive and oncogenic (Balkwill, 2002). As one of the major triggers of cancer initiation is reported to be chronic inflammation and since TNF-α is reported to induce inflammation, its role in promoting cancer progression is not a surprise. Supporting this idea, the knockdown of TNF α and TNF-α RI in mice has been reported to provide resistance to the induction of chemically induced carcinogenesis (Arnott et al., 2004; Moore et al., 1999). The production of TNF-α in the melanoma microenvironment has been related to autocrine secretion by melanoma cells itself and also from immune cells like monocytes and tumour associated macrophages (Bergenwald et al., 1997). Moreover secretion of TNF-α in the melanoma microenvironment has also been reported in tumours experiencing oxidative stress through the occurrence of tumour

associated macrophages (Lin et al., 2013). The reports suggest a significant presence of TNF α in the tumour microenvironment along with their prominent role driving cancer progression. Hence the possibility of a TNF-α-Twist1 axis driving melanoma drug resistance against BRAF and MEK inhibitors was tested.

On testing, we observed that TNF-α elevates Twist1 levels in BRAF mutant melanoma cell lines even in the presence of BRAF inhibitors (**Figure 10**).

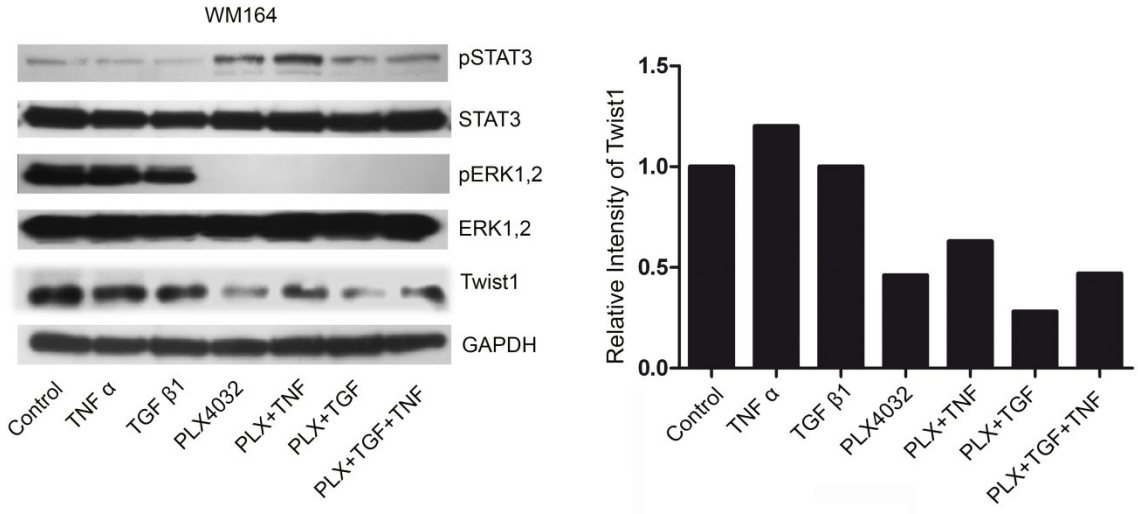


***Figure 10:* TNF α induces Twist1 expression even in the presence of BRAF and MEK inhibitors:** *Immunoblotting shows alterations of Twist1 levels in WM164 cells exposed to PLX4032 (1 μM), TGF β1(5ng/ml) and TNF α (10ng/ml) alone or in combination for 36hrs (left), Twist 1 bands were quantified and equalized to GAPDH as loading control (right)*

Combination of PLX4032 with TNF-α was observed to increase Twist1 expression when compared to PLX4032 only treatments. Quantification of the blots by image J software also showed a relative increase in the intensity of the tTwist1 band in the presence of TNF-α itself, whereas a combination of TGF-β1 with PLX4032 as observed before could considerably reduce Twist1 expression. This impact of TGF-β1 was observed to be partially reversible with the combination of TNF-α. The blots also suggested that the effect of TNF-α was independent of STAT3 and ERK phosphorylation.

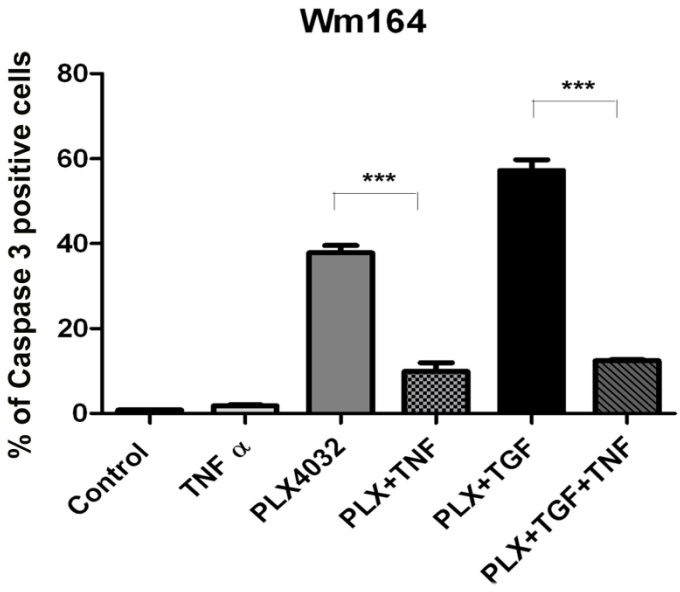


Figure 11: TNF α induce resistance PLX4032 even in the presence of TGF β1: *TNF-α(10ng/ml) induced rescue in WM164 cells treated with PLX4032 (1 μM)) also a combination of the same with TGF-β1(5ng/ml) , depicted as percentage of caspase 3 positive cells (***p-value <0.01- Student's t-test).*

Correspondingly TNFα substantially induced resistance against apoptosis induction by BRAF inhibitors (Figure 11). The rescue effect of TNF-α was quiet prominent even in the presence TGF-β1 suggesting that the rescue of Twist1 levels as observed in the immunoblots significantly affects the rescue of BRAF mutant cell lines towards BRAF inhibition.

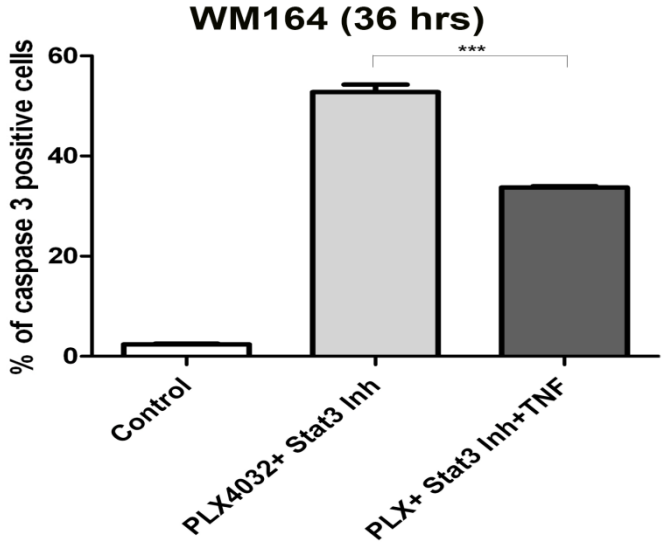


Figure 12: TNF α could induce resistance to a combination of PLX4032 with STAT3 inhibitor: *Percentage of caspase 3 activated cells in WM164 cells treated with PLX4032 and STAT3 inhibitor VII(1μM) in comparison to a combination of the same with TNF-α (***p-value <0.01- Student's t-test)*

Since the effect of TGF β1 was observed to be primarily directed through Stat3 phosphorylation, further the role of TNF α in inducing resistance to a combination of PLX4032 with STAT3 inhibitor was tested and the results observed was very much similar(Figure 12), suggesting that TNF-α leads to drug resistance through STAT3 and ERK independent signalling.

Further MEK inhibitors instead of PLX4032 were used obtaining similar results suggesting that TNF-α could invariably lead to resistance to both BRAF and MEK inhibitors.

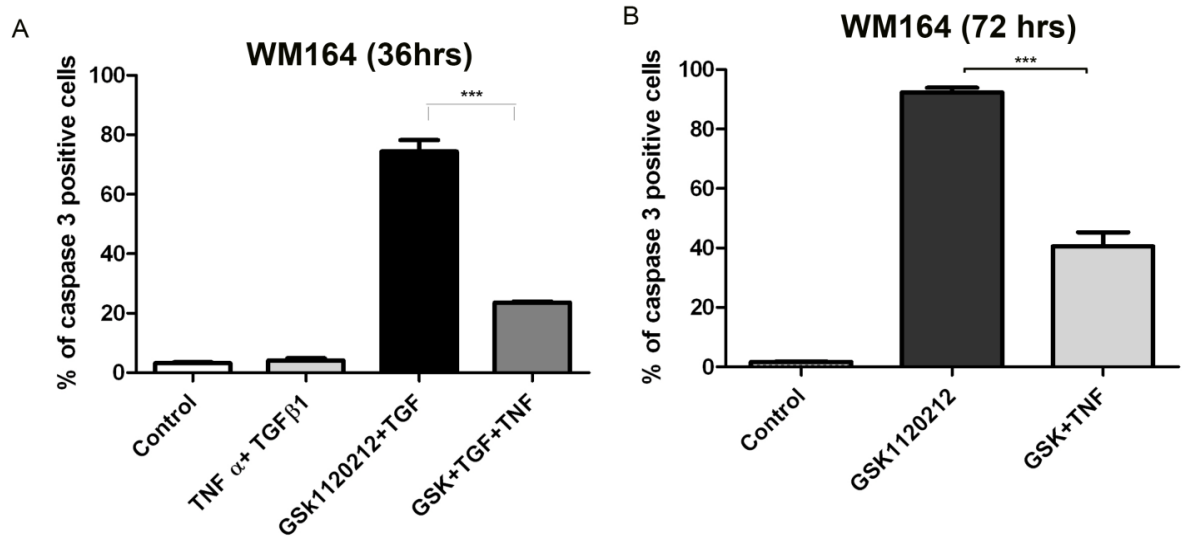


Figure 13. TNF α induces resistance GSK1120212 and its combination with TGF β1: *(A),(B)Percentage of caspase 3 positive cells in WM164 cells treated with TNF-α(10ng/ml), TGF-β1(5ng/ml) and GSK1120212(50nM) alone or in combination in comparison to cells exposed to DMSO (control) (***p-value <0.01- Student's t-test)*

The results were further confirmed in BRAF mutant cell lines SKMEL28 and WM9 which showed a very similar trend.

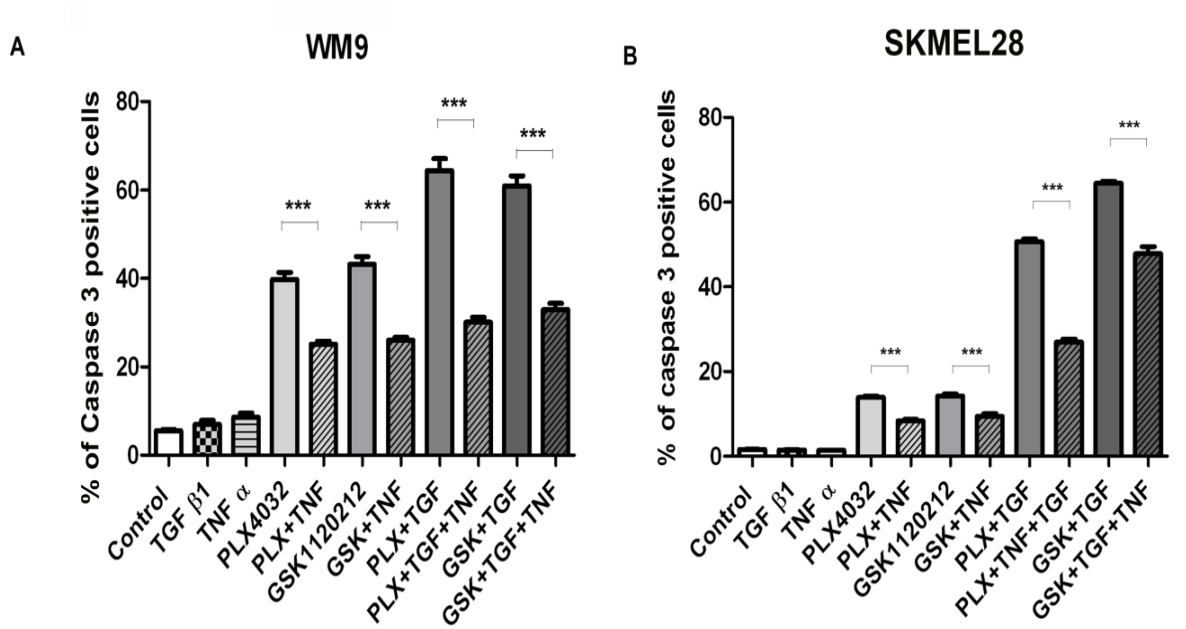


Figure 14. TNF α Induced rescue in WM9 and SKMEL28 cells : *TNF α (10ng/ml) induced rescue to PLX4032 (1 μM) and GSK1120212 (50nM) inhibitors and a combination of the same with TGF-β1(5ng/ml) in WM9(A) and SKMEL28 cells (B)(***p-value <0.01- Student's t-test).*

Since the results clearly indicated the role of TNF α in this context,Ifurther experiments were carried out to understand how much of these effects were mediated through Twist1. To probe this, previously described WM164 twist1 knockdown cells (WM164-sh-T) was used.

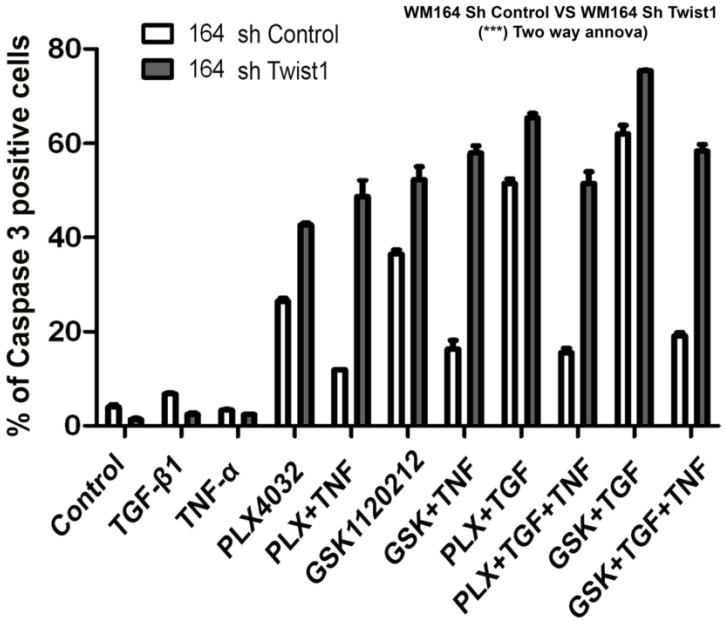


Figure15. Twist1 Knockdown leads to failure of TNF-α induced resistance in WM164 cells: *Comparison of TNF-α (10ng/ml) induced rescue in Twist1 stable knockdown cells (164 sh Twist1)(D) to control sh RNA (164 sh Control) transuded cells exposed to either PLX4032 or GSK1120212 in combination with TGF-β1(5ng/ml) . (***p-value <0.01- two-way ANOVA).*

WM164 control sh RNA transduced and WM164-sh-T transduced cells were exposed to PLX4032 or GSK1120212, in combination with TNF α, and observed that TNF α was unable to rescue BRAF and MEK inhibitor induced apoptosis in WM164-sh-T cells (Figure 15). To further confirm the results it was also tested whether the same is true with the combination of PLX4032 or GSK1120212 with TGF β1 and TNF α and observed similar results in either case, suggesting that Twist1 is necessary for the TNF α induced rescue effect. Further experiments were repeated in WM35 cell line, by generating WM35 Twist1 stable Knockdown cell line (Wm35 sh Twist1) to rule out a cell line specific effect in WM164,(Figure 16) , which further confirmed our previous results.

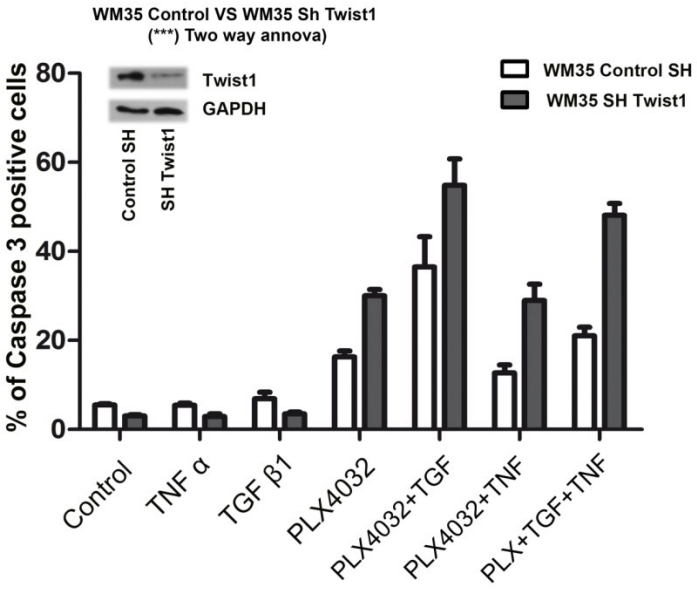


Figure16. Twist1 Knockdown leads to failure of TNF α induced resistance in WM35 cells: *Comparison of TNF-α (10ng/ml) induced rescue in Twist1 stable knockdown cells (WM35 sh Twist1) (D) to control sh RNA (WM35 control sh) transuded cells exposed to PLX4032 in combination with TGF β1 (5ng/ml). (***p-value <0.01- two-way ANOVA).*

Discussion

Together, results suggest that Twist1 can play the role of a central mediator in determining resistance against BRAF and MEK inhibitors in BRAF mutant melanomas. Twist1 is known to play an important role in Melanoma progression by its activation through the BRAF-ERK signalling (Weiss et al., 2012b). Also activation of Twist1 in melanocytes has already been reported to be a natural response to BRAF mutations which leads to neoplastic transformation (Caramel et al., 2013). These findings are in line with various other reports which clearly underscore the importance of Twist1 in cancer progression (Ansieau et al., 2008). Correspondingly, the role of Twist1 in EMT, cancer cell invasion and metastasis is also well documented (Eckert et al., 2011; Yang et al., 2004). However, the impact of Twist1 with regard to melanoma drug resistance and specifically BRAF and MEK inhibitor resistance has not been investigated so far. My observations indicate its prominent role in melanoma drug resistance to molecular targeted inhibitors, in support of previous reports that have described a substantial contribution of Twist1 to drug resistance in other form of cancers (Kwok et al., 2005; Li et al., 2009; Shiota et al., 2010)Since Twist1 activation can be mediated by multiple pathways activation of any of the intrinsic survival pathways with or without extrinsic stimulation which contribute to Twist1 expression, like Stat3 or TNF-α signalling seems to sufficient to induce drug resistance. Hence in this context the factors in the tumour microenvironment could be prominent deciding factors in the response of patients to BRAF and MEK

inhibitors. A higher level of TGF β1 in tumor microenviornment may favour the response in patients to BRAF or MEK inhibitor, but TNF-α might override this effect by increasing Twist1 expression through parallel pathways. TNF-α is expressed not only by cancer cells but also by myeloid cells and TAMs associated with the tumor microenvironment (Kim et al., 2009b; Pollard, 2004). Myeloid cells in particular are migrating to sites of tissue damage, a situation, which is also found in tumour environments treated with cytotoxic drugs (Nakasone et al., 2012; Shree et al., 2011). Together this points to the importance of TNF-α induced drug resistance in the context of anti-cancer therapies.

Based on my findings and reported by others ((Weiss et al., 2012b, Caramel et al., 2013) Twist1 is a drugable target not only because of its influence in cancer progression but also because of its contribution to drug resistance. In this respect combinations with molecular targeted inhibitors like BEAF and MEK could be thought of. A more strategic approach suggests the combination with STAT3 inhibitors. This kind of approach holds promise as recent innovations aimed successfully in targeting STAT3, which was currently considered to be “undruggable” (Sen et al., 2012). From another point of view inhibitors of TNF α signalling have been already developed and many of them are undergoing clinical trials for various cancers (Huseyin TE Ozer, 2010; Madhusudan et al., 2004). Combinations of some of these potential drug targets with BRAF or MEK inhibitors hold considerable promise in producing more favourable patient response. While that being said, further testing and in vivo analyses have to be carried out to better assess these strategies.

1.3 Materials and methods

(1.3.1) Antibodies

Primary antibodies against Slug ([G18], Santa Cruz Biotechnology, Santa Cruz, CA), Snail (Abcam, Cambridge, UK), Twist1 ([Twist2C1a], Abcam), ERK1/2 total and phosphor- ERK 1/2 (Cell Signalling), Active Caspase 3 (cell signalling), STAT3 and STAT3 Tyr705 (Abcam) were used. GAPDH and Beta actin were purchased from Santa Cruz biotechnology. Goat anti mouse secondary antibody was purchased from DAKO and goat anti Rabbit again from Santa Cruz Biotechnology.

(1.3.2) Inhibitors and cytokines

The BRAF inhibitor PLX4032 and MEK inhibitor GSK1120212 were obtained from Selleckchem (Houston, TX). The STAT3 Inh VII was obtained from Calbiochem. TGF-β1 receptor inhibitor SB431542 was obtained from Sigma Aldrich. Human active TGF-β1 was purchased from Abcam and TNF-α from Sigma Aldrich.

(1.3.3) Immunoblotting

Immunoblotting was performed essentially as outlined in Damm et al., 2010, Koefinger et al., 2011, and Wels et al., 2011. Whole cell lysates were generated using RIPA buffer (Sigma). Protein concentrations were measured using the Bradford protein assay (BioRad, Hercules, CA). Biod Rad wet tank blotting systems were used to perform immunoblotting. Samples are loaded and separated on SDS-polyacrylamide gels(10%), transferred to polyvinylidene difluoride (PVDF) membranes and probed with specific primary antibodies overnight at 4^{o}c. To detect the signal, peroxidase-conjugated secondary antibody was added and incubated for 4 hours in room temperature. Proteins were visualized using ECL reagents (Amersham, Piscataway, NJ) and exposed to X-ray film (Kodak). The quantification of bands were done by Bio Rad quantity one software by background subtraction method, or with Image J analysis.

(1.3.4) Crystal Violet Colony assay

Cells were continuously exposed to the respective inhibitors and media was changed every three days ‘till the end of the experiment. The cells were then washed with PBS, fixed with formaldehyde and stained with 0.05% Crystal violet solution. For quantification cells were eluted of the stain with 10% acetic acid for 2 hrs and the optical density was read at 590nM by a microtiter plate reader.

(1.3.5) Caspase 3 apoptosis assay

Intracellular Caspase 3 was measured using Caspase-3, active form, mAb Apoptosis Kit PE, following the user manual (BD Pharmigen). Briefly the cells were washed with ice cold PBS twice and further fixed and permeabilized by the BD Cytofix/Cytoperm™solution for 20 mins. The fixed cells were rewashed twice with BD Perm/Wash™ provided and stained with PE-conjugated Monoclonal Rabbit Anti-

Active Caspase-3 Antibody for half an hour in 1:10 ratio with BD Perm/Wash™ buffer. The cells were further washed with 1ml of BD Perm/Wash™ buffer and the percentage of positive cells were analysed by BD LSR II Flow Cytometer. The experiments were done in triplicates or in duplicates and graphs were plotted with mean and error bars describing standard deviation from the mean.

(1.3.6) Viral constructs and transduction

pcDNA4-hTwist was a kind gift of Dr. Carlotta Glackin (Department of Neurosciences, Beckman Research Institute of City of Hope, Duarte, CA, USA) and was cloned into the PLXRN vector. PG-V-RSP sh Twist1 was bought from Origene. Retroviruses are produced by two-plasmid co-transfection of GP-293 cells with above mentioned constructs and the envelope protein-coding plasmid pVSV-G using the ProFection® Mammalian Transfection System (Promega). (Damm et al., 2010; Koefinger et al., 2011; Wels et al., 2011).Wm35 sh Twist1 stable cell line was generated using sh Twist1 lentiviral particles bought from Santacruz biotechnology.

Cells before 24 hours of transduction were plated in 6 well plates so that they reach 60% confluency on the day of transduction. The cells were to exposed 8µg/ml of polybrene complete media with corresponding lentiviral particles for 24 hours. Further they were selected with corresponding antibiotics. For PLXRN and PG-V-RSP sh Twist1 constructs, G418 (Gibco) (400µg/ml) was used over 2 weeks period for selection. For sh Twist1 lentiviral particles purchased from Santa Cruz biotechnology, puromycin at 10µg/ml was used for selection over a week.

(1.3.7) MTT assay

20000 Cells were seeded in a 96- well culture plate in triplicates. Cells were allowed to attach for the next 12 hrs followed by treatment for a 72 hrs period. The cells were subsequently incubated with MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) reagent at 37C° for 3 h and absorbance was measured at 570nm by a microtiter plate reader. Experiments were done in triplicates.

CHAPTER 2

Drug Induced Cancer Stemness And Acquired Drug Resistance

(2.1)Introduction

(2.1.1)Cancer stem cells

The cancer stem cell theory argues that a very minor percentage of the parent cancer population consists of stem cells which are the major drivers of the disease. It has been argued that cancer stem cells are equivalent to normal stem cells or progenitor cells which are capable of self-renewal and asymmetric cell division (Pardal et al., 2003). Hence the model points to a possibility of cancer cells arising from a mutated stem cell population (Figure 17).

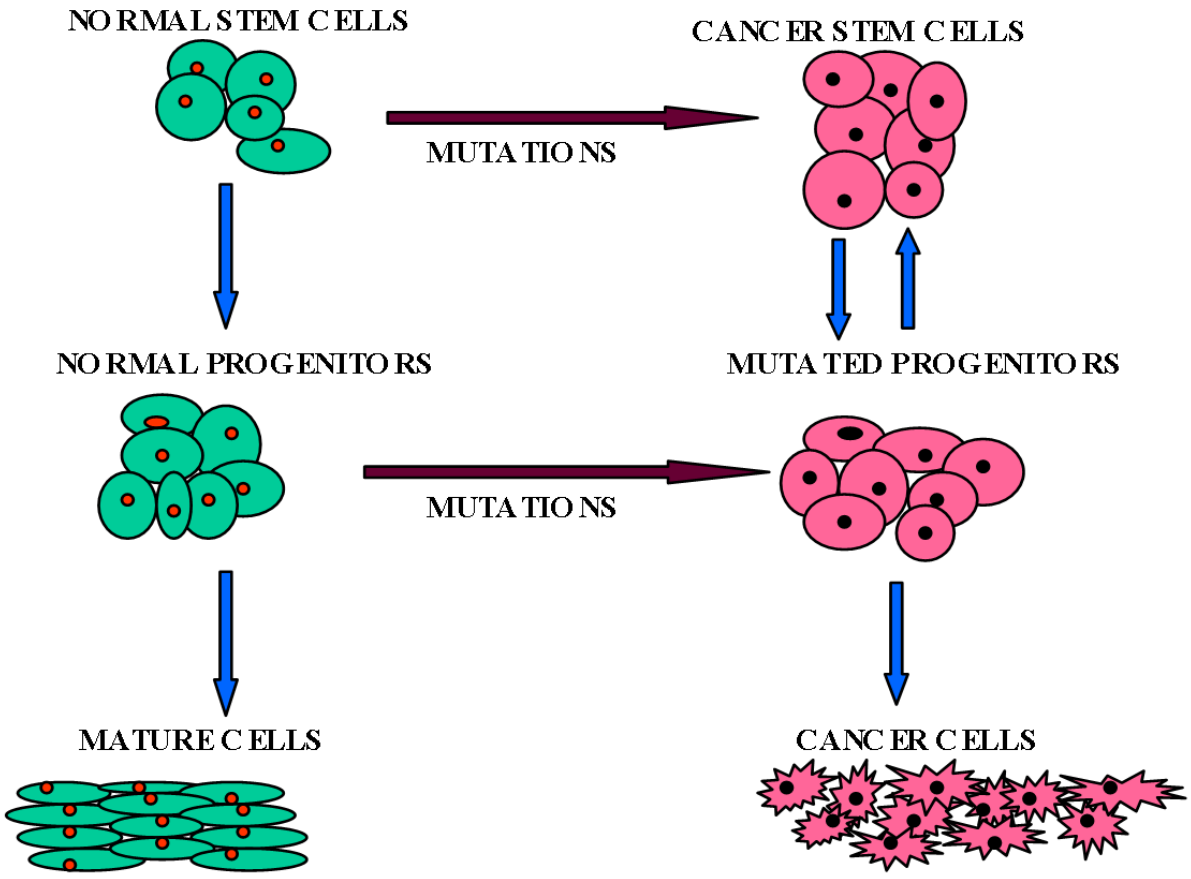


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Figure 17. Cancer Stem cells and tumour initiation: *A possible model for the origin of cancer stem cells leading to tumour formation. Adapted from(Sagar et al., 2007)*

Due to these characteristics they have also been linked to the initiation of tumour formation and have been indicted to have a high tumourogenic potential. The initial proof of this concept was observed in acute myeloid leukaemias where a rare subpopulation of CD34+, CD38− cells were able to form large tumours in SCID mice(Bonnet and Dick, 1997).

This concept was extended to solid tumours like breast cancer where a subpopulation of CD44+ ,$CD24^{-/low}$ cancer initiating cells were identified (Al-Hajj et al., 2003). Similar findings were also observed in brain tumours (Singh et al., 2004), colon cancers (O'Brien et al., 2007) and pancreatic cancers (Hermann et al., 2007) where the population was identified with a CD133 marker. In hepatocellular carcinoma (HCC) the marker was identified to be the epithelial cell adhesion molecule (EpCAM)(Yamashita et al., 2009). A similar population was described in melanomas which were identified using ABCB5 (Schatton et al., 2008) and CD271 (Boiko et al., 2011) expression. Also a recent addition to this group of markers is the increase in ALDH activity which has been reported in multiple cancer types including leukaemia, breast cancer, lung cancer, Melanoma, liver cancer etc(Marcato et al., 2011a), providing a candidate for a potential universal marker for identifying cancer stem cells. The isoforms of aldehyde dehydrogenase leading to ALDH activity, however, have been reported to be diverse in different cancer types. Altogether the current literature shows enough data which supports this theory of cancer stem cells attracting a lot of further research to understand how this model can be used for therapeutic advantages.

(2.1.2)Cancer stem cells and drug resistance.

It has been proposed that the cancer stem cell model has wider implications with respect to drug resistance. The core of this hypothesis is that the small subpopulation of cancer stem cells in the parent tumour are drug resistant and hence survive the drug exposure leading to a relapse of the disease by reforming the parent tumor (Figure 17A) or by achieving mutations to form resistant tumours (Figure 17B). The basis of this idea stems from the finding that drug exposure at cytotoxic concentrations is widely observed to lead to survival of a small population of cells which has been described to express previously mentioned stem cell markers. This phenomenon has been observed in multiple cancer types including ovarian cancer (Rizzo et al., 2011), melanoma (Chartrain et al.) and breast cancer (Calcagno et al., 2010). The idea has gained a lot of popularity as many of the cancer stem cell subpopulations have been reported to express drug efflux proteins. In melanoma for example the marker of the tumour initiating population ABCB5 is a

drug efflux gene which has been described to be capable of effluxing doxorubicin, a chemotherapeutic drug(Frank et al., 2005).

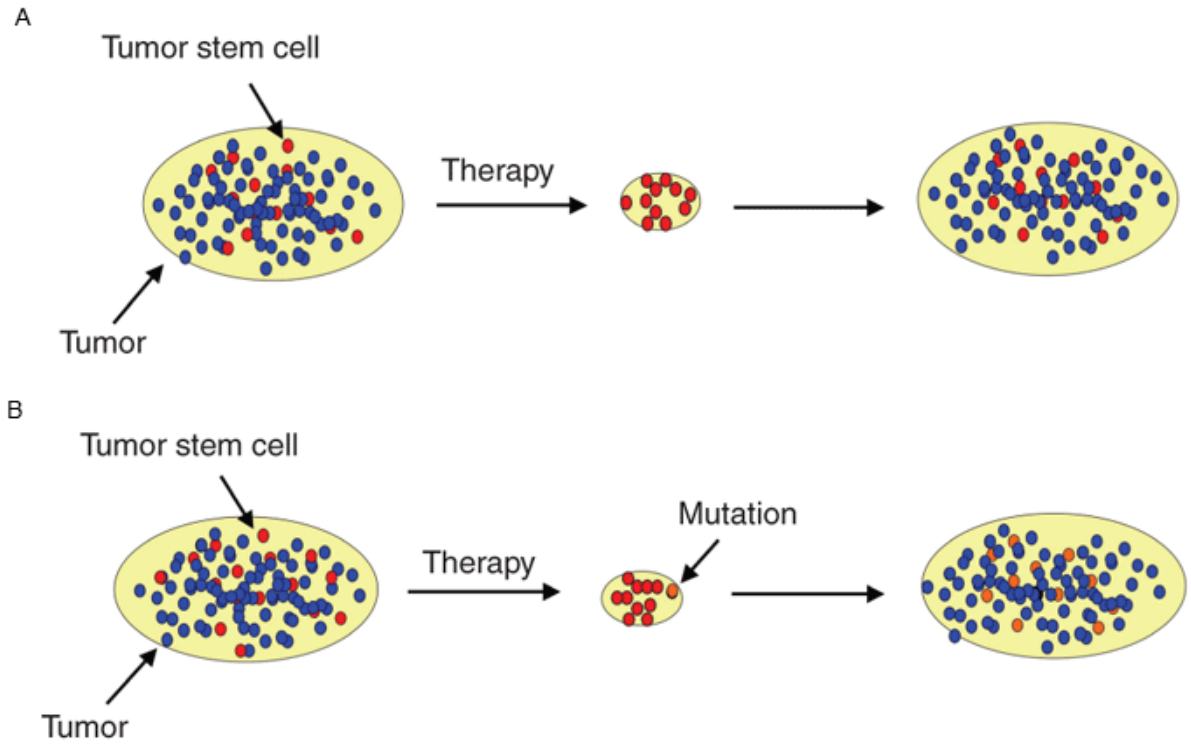


Figure 18: Relapse in Tumour through Cancer stem cells. *Cancer stem cell model predicting the survival and selection of stem cells during the course of therapy which further leads to the relapse in disease by itself(A) or gets mutated to induce the formation of a resistant tumour(B). Adopted from(Moitra et al., 2011)*

Similarly multiple drug efflux capable genes like ABCB1,ABCG2 and ABCC1 which all use energy from ATP hydrolysis to efflux and transport drugs, has been described to be expressed in multiple cancer stem like cells(Dean et al., 2005). Hence a lot of interest and research have been bestowed in to targeting these drug efflux genes to enhance the response to drugs.(Gottesman et al., 2002). However clinical trials with many of these drug combinations failed because of various pharmacokinetic issues(Dean et al., 2005).

Due to the advancements in molecular biology techniques and better understanding of driver mutations, genes and pathways, the basic therapeutic strategy is currently undergoing a major overhaul from generalised cytotoxic chemotherapeutic drugs to targeted molecular inhibitors and antibodies. However the enrichment of cancer stem

cells even in the end of these strategic regimes still remained a major problem, like in case of drug treatments with Erlotinib® a targeted inhibitor against EGFR administered to patients having EGFR mutated cancer (Corominas-Faja et al., 2013; Ghosh et al., 2012). Eventhough these cells have been reported to have drug efflux capabilities no significant efflux of Erlotinib was observed, suggesting that drug efflux is not directly contributing to drug resistance in these cells.

These observations led to new studies which came up with a slightly different model based on a slow cycling transient population of cells which are drug resistant by its very nature surviving very high drug dosages ensuring the survival of the cancer population. The model proposed that a very small percentage (~0.1-5%) of the parent cancer population has a distinctive chromatin state which they acquire and loose randomly, are inherently multiple drug resistant.

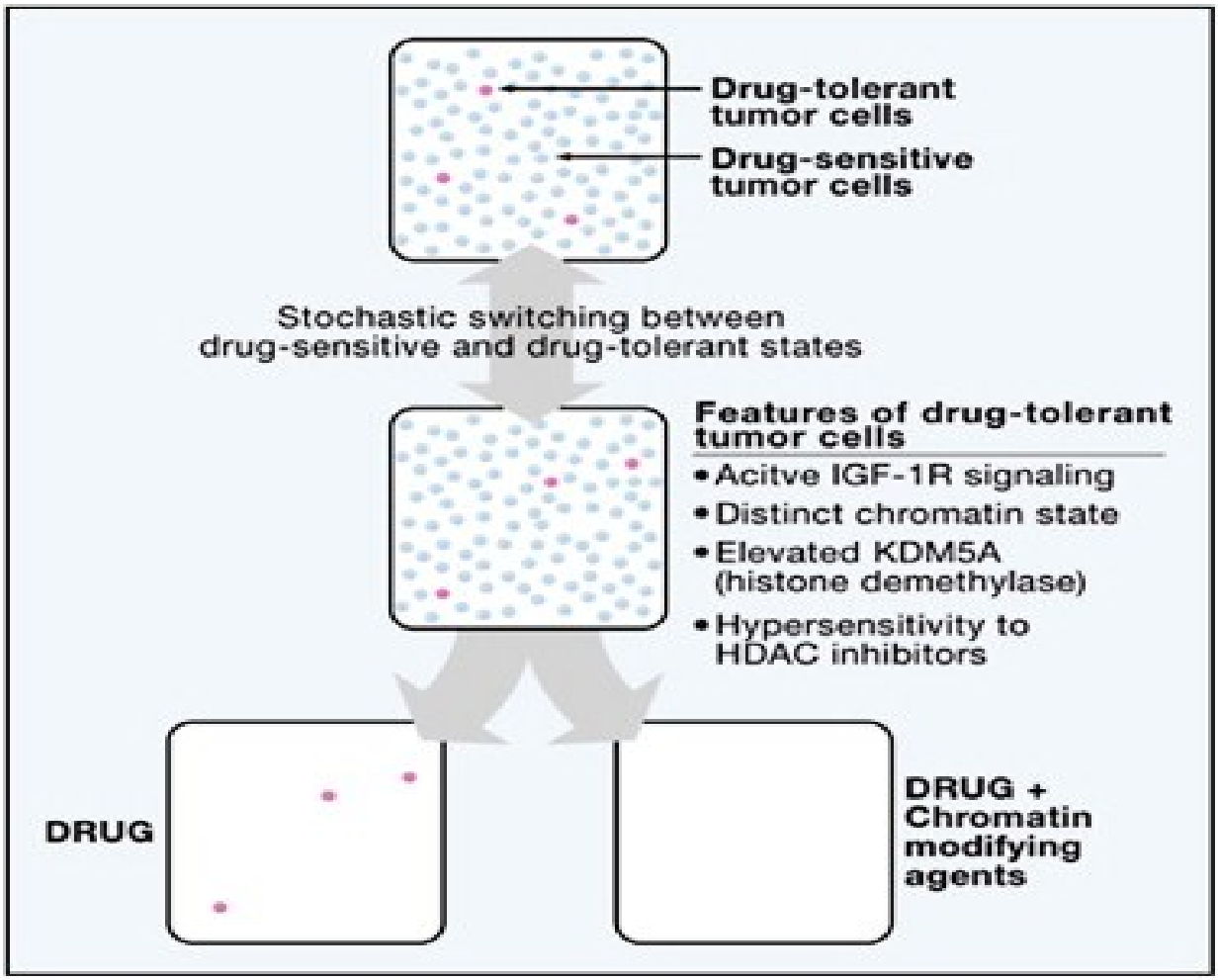


Figure 19: Chromatin Mediated drug resistance model: Model explaining survival of drug resistant cells with altered chromatin state which they randomly acquire according to Sharma et.al, 2010. Adapted from (Sharma et al., 2010).

The distinctive chromatin state was characterised by the loss of H3K4me3 and increase in the H3K4 demethylase KDM5A. Even though the nature of random origin of these cells remained unexplained much like the origin of cancer stem cells, the study showed much evidence that targeting the altered chromatin state by using HDAC inhibitors, the IGF1R pathway or KDM5A itself was sufficient enough to eliminate these cells (Sharma et al., 2010). They also showed that the model can be applied to multiple cancer types and similar results have been reproduced in melanomas where a KDM5B positive subpopulation was shown to exhibit similar characters (Roesch et al., 2013). In this paper it was suggested that targeting mitochondrial signal pathways in combination to be a better strategy than targeting using HDAC inhibitors and IGF1R inhibitors alone. Together these studies suggested that a small percentage of the parent cells, representing a subpopulation are capable of exhibiting drug resistance leading to their selection and survival which on the long run leads to acquired drug resistance.

(2.2)Results

(2.2.1)Multiple drug tolerant Cancer Stem Like cell formation can be induced by drug exposure

A significant amount of literature in cancer biology suggests that drug resistant subpopulations pre-exist and are selected during the process of drug treatment. If this holds true, it could be expected that a certain percentage of parent cells is constantly surviving drug exposure under any condition. From the previous experiments as mentioned in Chapter 1 it became clear for me that the number of cells surviving the initial drug exposure can tremendously vary due to both intrinsic and extrinsic factors. The best example of this was the long term survival assays that was performed in WM164 cells with different combinations of DMSO, PLX4032 (1μM), TGF-β1 (5ng/ml), SB431542 (10μM) (a inhibitor of TGF β1 receptor) and TNF-α (10ng/ml). In accordance to my previous results I observed that a combination with TGF β1completely eradicated the parent population at day 12, whereas

exposure to SB431542 or TNF α significantly increased the number of surviving cells (Figure 20).

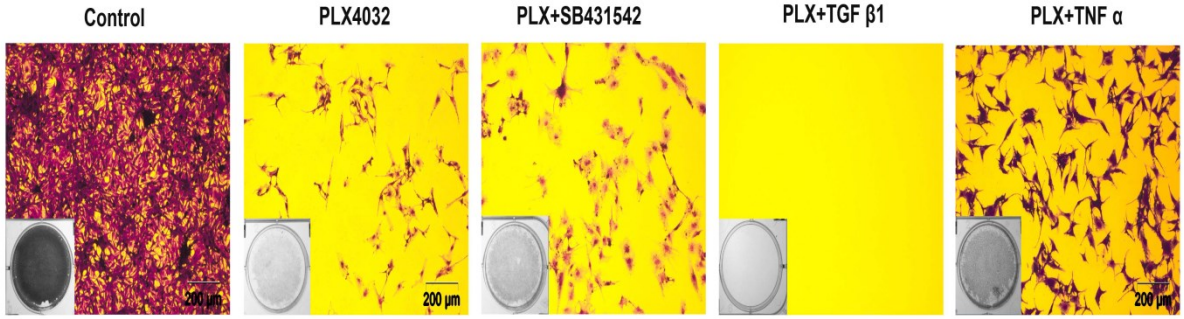


Figure 20, Long term survival assays: *Crystal violet staining of surviving cells after 12 days of exposure to PLX4032, PLX4032+SB431542 (PLX+SB431542), PLX4032+TGF β1 (PLX+TGFβ1) or PLX4032+TNF (PLX+TNF)*

Since it is quite logic that a combination of drugs enhances the effect of a single drug thus leading to more cell death as observed by combining PLX4032+TGF β1 . In that case if it is assumed that the percentage of a pre-existing drug resistant stem cell like population persists, roughly representing between 0-5% of the parent cells it would be expected that the remaining population that survived 12 days of drug exposure should be still susceptible to a higher dosage of the same drug or a combination therapy like PLX4032+TGF β1, which in case of WM164 cells almost completely eradicated the parent population (Figure 20). Or on the other hand eradication of the entire parent population suggests that the combination of PLX4032 with TGF β1 has eliminated the pre-existing drug resistant stem like population, if any. Therefore I tested this by isolating 12 days PLX4032 exposed WM164 cells and subjected them to a combination of PLX4032 with TGFβ1 or GSK1120212 with TGFβ1. Interestingly it was observed that these cells were no longer sensitive to any of the combination therapies (Figure 21 A). Prolonged exposure for another 12 days to PLX4032 and TGF β1 did not induce any significant difference in cell survival (Figure 21 B).

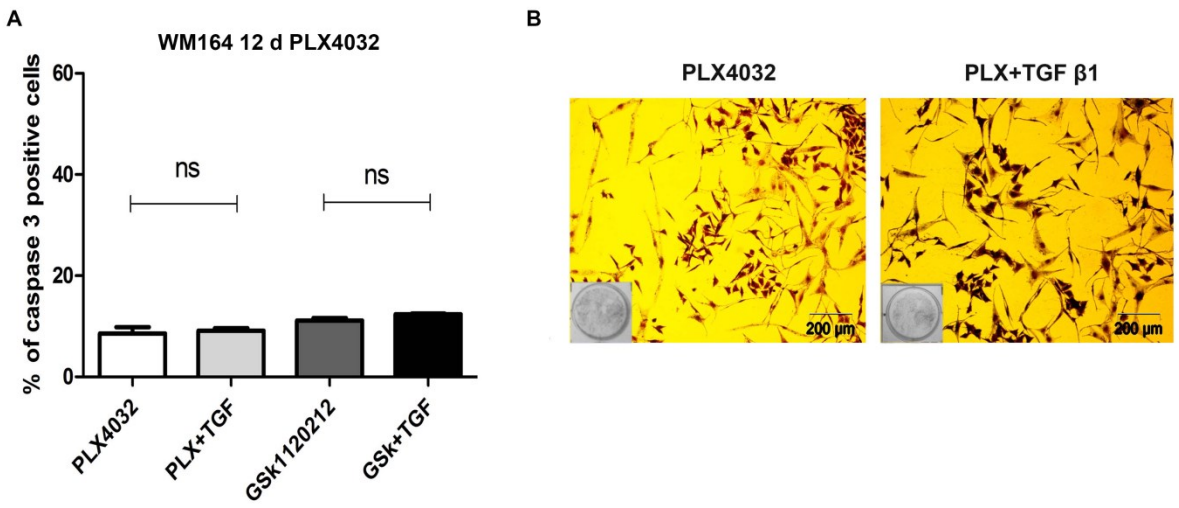


Figure 21; WM164 cells surviving 12 Days of PLX4032 show a remarkable drug resistance capability: (A) Percentage of caspase 3 activity of WM164 cells exposed to PLX4032 at 1µM for 12 days and exposed again to PLX4032 or GSK1120212 alone or in combination with TGFβ1 as analysed by flow cytometry. (B) WM164 cells exposed to PLX4032 at 1µM for 12 days, and further exposed for 12 days by combining PLX4032 with TGF β1 in comparison to PLX4032 only treatment. The cells were stained with crystal violet at the end of the experiment and pictures were taken under the microscope.

These results raised a very fundamental question to the existing models of cancer drug resistance including the stem cell model or chromatin mediated drug resistant slow growing tumour subpopulation model, both of which argue for the pre-existence of a fixed or very small number of drug resistant cells in the parent population. My results, however, suggested that the survival of drug resistant cells might vary according to the effectiveness of the initial insult, while the cells surviving this initial dose emerge as drug resistant cells. It also suggested that the very exposure to the drugs is inducing a transition in the cells providing them with drug resistant capabilities independent of a pre-existing subpopulation.

To test for this hypothesis I exposed $1x10^6$ BRAF mutated WM164 melanoma cells (WM164) to various concentrations (250nM, 500nM, 1µM, and 10 µM) of the BRAF inhibitor PLX4032 for a period of 12 days. The idea again was to look at the number of cells surviving after each dosage, in order to select a dosage where there is no significant cell death in the parent population. This dosage should be sublethal, a condition where there is no significant selection pressure and the effect of continuous exposure to such moderate drug concentrations yields a drug resistant population. The experiments were done in triplicate(s) and after 12 days 2 of the triplicates were subjected to casy cell counting whereas the 3rd was subjected to

crystal violet staining for representative pictures (Figure 22 A, B). The number of cells surviving the exposure ranged from 4.51% (+/-0.35) at 10µM to 99.5% (+/-1.48) and 156.5% (+/- 13.43) at 500nM and 250nM, respectively, indicating that after 12 days of 500nM and 250nM PLX4032 the number of surviving cells was equal or marginally below the parent population at day 0 (Figure 1A, B), suggesting that no significant selection pressure could have occurred under these conditions. Based on this observation it could be suggested that if only a limited number of cells, as demonstrated for 10µM, are inherently drug tolerant to high drug concentrations of PLX4032 it would be expected that at lower dosages, which lead to the survival of almost the entire population of cells, still shows sensitivity towards higher dosages of PLX4032. Therefore this was probed by further exposing the 250nM and 500nM PLX4032 treated cells to 10µM PLX4032 for the next 12 days. Interestingly, , cells exposed to lower concentrations of PLX4032 were tolerant to even very high concentrations of the drug, with more than 100% of the cells surviving drug exposure (Figure 22 A,B). This suggests that a low drug dose instigates an early response in the surviving population leading to drug tolerance hence the population was defined as induced drug tolerant cells (IDTCs).

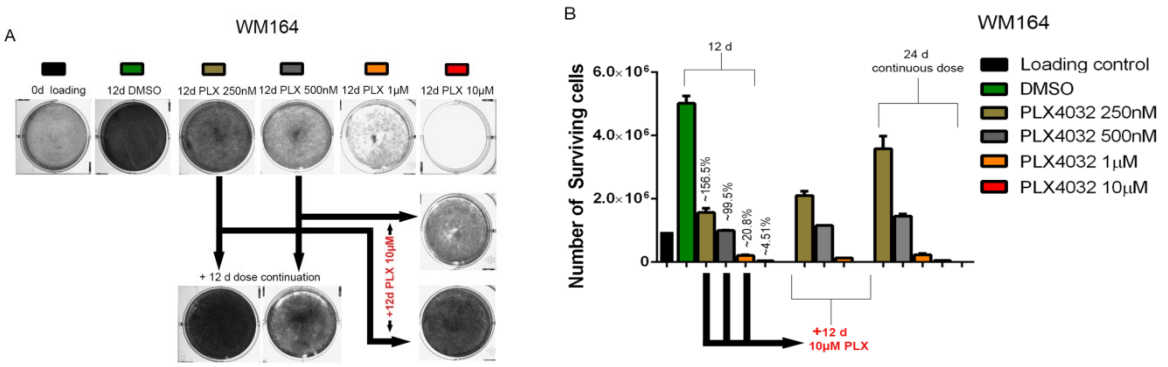


Figure 22: Moderate drug concentrations induce drug resistance to higher drug doses. *1x10^6 WM164 cells were plated in triplicate for each time point and drug dosage, and treated with DMSO and PLX4032 (250nM, 500nM, 1µM and 10µM) for 12 days. One of the 3 triplicates were subjected to crystal violet staining to take a representative image (A) and the number of viable cells were counted with a casy cell counter from two experimental duplicates shown as bar graphs. Error bars represent the standard deviation from the mean (B). Further 500nM and 250nM treated cells were subjected to 10µM PLX4032 for another 12 days, whereas a separate set of cells where given continuous 250nM, 500nM for a 24 days period as a secondary control to understand the growth dynamics of WM164 in each dosage for the entire experimental period. After 24 days one of the triplicates from various experimental conditions were stained with crystal violet (A) and again the number of viable cells in the duplicates were analysed by casy counting (shown as bar graphs). Error bars represent the standard deviation from the mean (B).*

We further tested whether the exhibited drug tolerance was specific for PLX4032 or generic in nature by exposing 250nM and 500nM generated WM164 IDTCs to the MEK inhibitor GSK1120212 or cisplatin for 2 days. These cells showed an even significantly lower sensitivity to other drugs, suggesting IDTCs represent a multiple drug tolerant state (Figure 23).

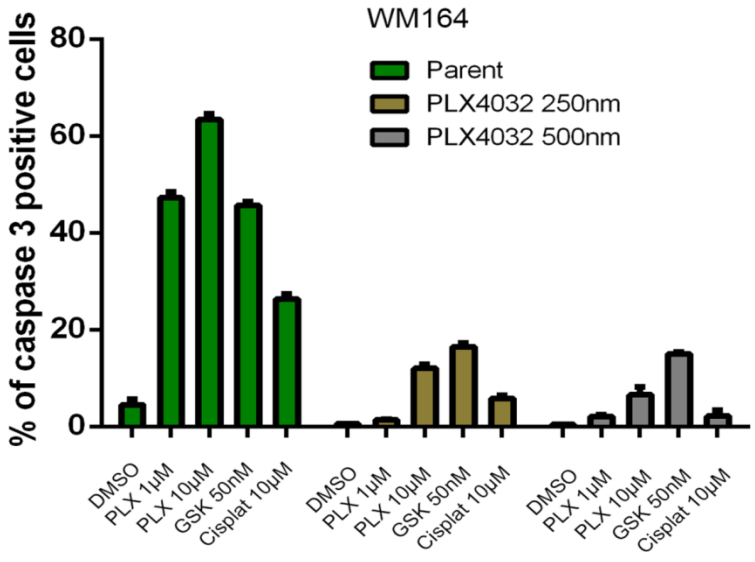


Figure 23 : Induced Drug Tolerant Cells (IDTCs) exhibit multiple drug tolerance*. WM164 PLX4032 250nM and 500nM IDTCs (WM164 250 IDTC, WM164 500 IDTC) were exposed to PLX4032 1µM, 10µM, GSK1120212 50 nM and Cisplatin 10 µM for 48 hours and cells were analysed by flow cytometry for the percentage of Caspase 3 positive cells. Error bar represents standard* ***deviation from***

The experiment was repeated in the BRAF mutant A375 melanoma cell line at 100nM and 250nM PLX4032, which was again observed to be a sublethal dosage for that cell line. Further treatment with higher dosages of PLX4032, on the cells that survived 12 days of sublethal dosage showed no significant response. Similar to what was observed in WM164 IDTC, further treatment with GSK1120212 or Cisplatin did not yield any significant induction of apoptosis when compared to the parent population (Figure 24 A, B)

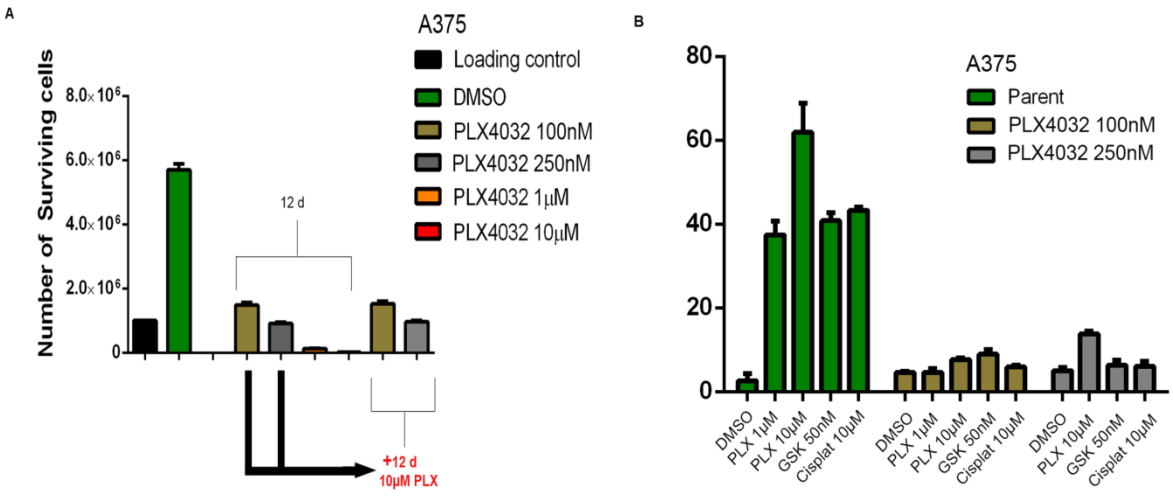


Figure 24: A375 BRAF mutant melanoma cells show a similar behaviour at low and moderate drug concentrations like WM614 drug doses*. 24 day experiments were essentially repeated in A375 cells with PLX4032 at 100nM, 250nM, 1μM and 10μM in duplicates and viable cell count depicted as bar graphs. Error bars represent the standard deviation from the mean (A). A375 PLX4032 100nM and 250nM IDTCs were subjected to PLX4032 at 1μM, 10μM, GSK1120212 at 50nM and Cisplatin at 10μM for 48 hours and cells were analysed by flow cytometry for the percentage of caspase 3 positive cells (B). Error bars represent the standard deviation from the mean*

Moreover the phenomenon of multiple drug tolerance was reversible, as a 7 days drug holiday provided to IDTCs was sufficient to re-sensitize the cells to both PLX4032 and GSK1120212 suggesting that the tolerant state was induced by the drug exposure itself.

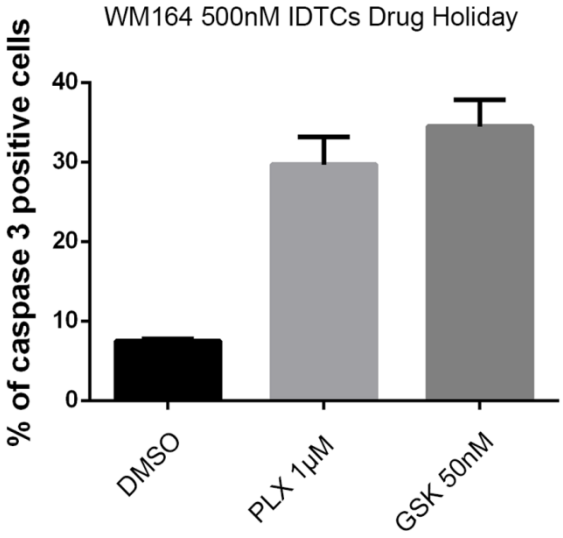


Figure 25: Induced Drug tolerance is reversible *.WM164 500nM IDTCs were provided a 7 days drug holiday and resubjected to1μM and 10μM PLX4032. Tthe percentage of caspase 3 positive cells was analysed by cell sorting. Error bars represent the standard deviation from the mean.*

To understand the reason behind the drug tolerance to PLX4032 exhibited by IDTCs, western blot analyses of the key survival pathways MAPK-ERK and AKT were carried out. WM164 IDTCs showed highly active ERK, MEK and AKT signaling (Figure 26 A). Time point analyses of WM164 exposed to 500nM PLX4032 revealed an increase in both phosphorylated AKT and MEK-ERK at day 8 after exposure, which further peaked at day 12 (Figure 26 B). This suggests that only persistent exposure for eight days initiates the transition into an IDTC state in WM164 cells. Even though the reactivation of the ERK pathway is an approved mechanism for drug resistance to PLX4032, it cannot sufficiently explain the same for other drugs like cisplatin or GSK1120212.

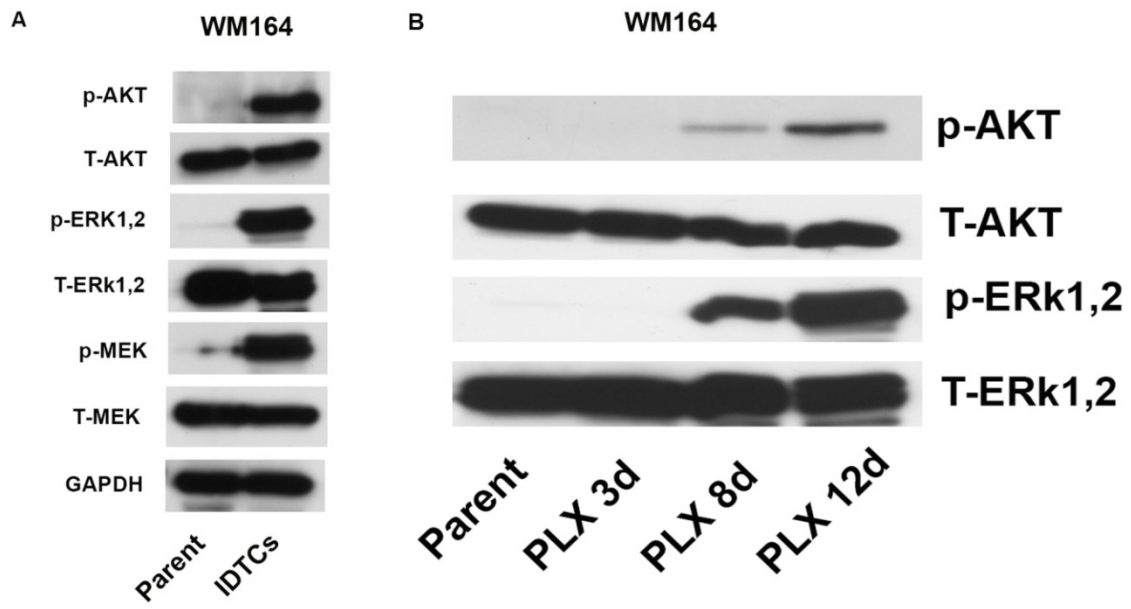


Figure 26: IDTCs show reactivation of ERK and AKT signalling*. (A) Lysates of Wm164 500 IDTC and parent cells were subjected to immunoblotting to detect phosphorylated ERK1,2(p-ERK1,2(Thr202/Tyr204)) , total ERK(T-E RK1,2), Phosphorylated MEK1,2(p-MEK Ser217/221), total MEK1,2(T-MEK), Phosphorylated AKT (p-AKT (Ser473)), Total AKT (T-AKT) and GAPDH for loading control(B)Time point analysis of emergence of ERK and AKT phosphorylation on I DTCs by subjecting to immunoblotting to detect phosphorylated ERK1,2 (p-ERK1,2 (Thr202/Tyr204)), total ERK(T-E RK1,2), Phosphorylated AKT (p-AKT (Ser473)), Total AKT (T-AKT)*

Hence we further characterized IDTCs by whole genome wide gene expression analysis and compared expression profiles to the parent populations with the help of molecular biology core facility of the ZMF.

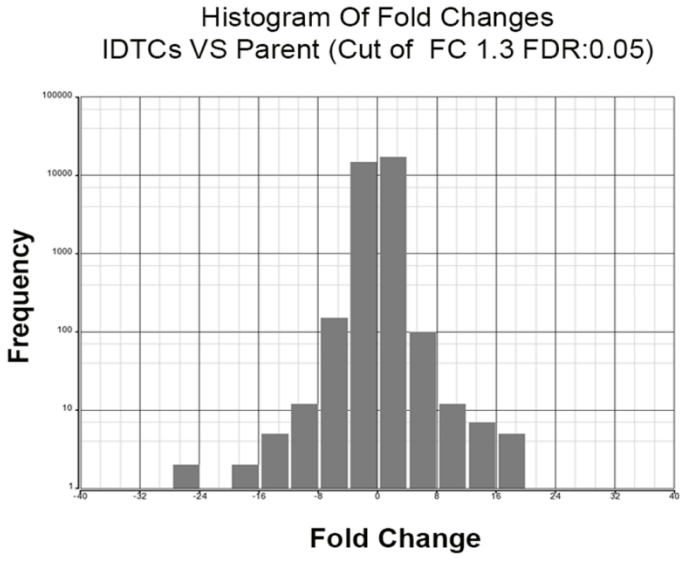


Figure 27: Changes in Global Gene Expression. *Histogram representing the frequency of genes showing the representative fold change difference in WM164 IDTCs vs the parent cells with a p <0.0180382 (=passing multiple testing of FDR of 5% and a minimum fold change between 1,3 to -1,3*

We observed substantial differences in global gene expression between the WM164 parent cells and IDTCs (Figure 27). Distinctively, a significant increase in the expression of various stem cell markers like CD271, ABCB5, SOX10 (Boiko et al., 2011; Civenni et al., 2011; Mohamed et al., 2013), CD44 (Ponti et al., 2005), SOX2 (Herreros-Villanueva et al., 2013), and the EMT inducer SOX4 (Tiwari et al., 2013) in IDTCs was observed. The cells also showed increases of various drug efflux genes like ABCA5 (Alla et al., 2012), ABCB8 (Elliott and Al-Hajj, 2009) and ABCB4 (Duan et al., 2004) (Figure 28A, B).

A

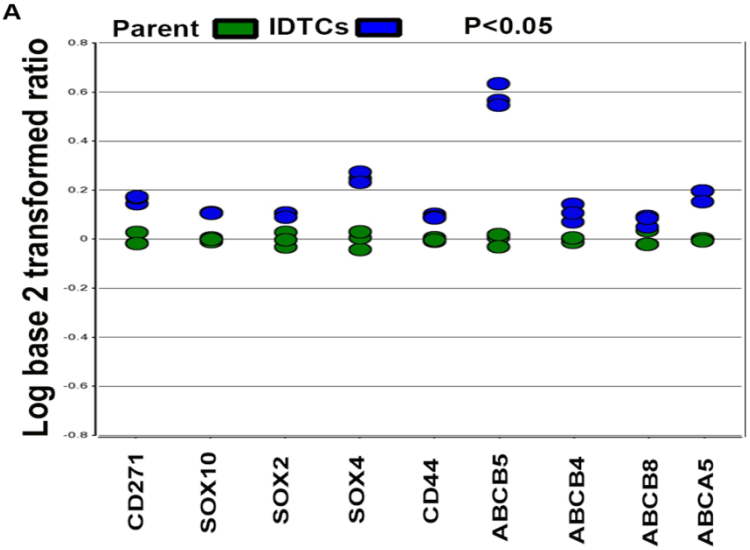


Figure 28: IDTCS show an increased expression of stem cell genes. *(A)Selected Stem cell genes from Microarray analysis comparing the Log 2 transformed ratio of WM164 IDTCs (Blue) to that of parent population (green) where the mean value of parent samples was equated to zero, and each dot represent one of the triplicates.(B) Table showing the genes represented in (A) , Their transcript ID, Gene Symbol REF SEQ ID, p- Value and Fold change.*

B

Transcript ID	Gene Symbol	REFSEQ	P Value IDTCs VS PArent	Fold Change IDTCs Vs Parent
8008201	NGFR	NM_002507	3.08129E-05	1.84701
8075992	SOX10	NM_006941	3.37383E-06	1.79705
8084165	SOX2	NM_003106	0.00227213	1.342
8117165	SOX4	NM_003107	1.17396E-05	2.65369
7939341	CD44	NM_000610	1.55084E-06	1.7683
8131682	ABCB5	NM_178559	1.21477E-06	3.95822
8140752	ABCB4	NM_000443	0.00110056	1.2873
8137332	ABCB8	NM_007188	0.0093655	1.30422
8018038	ABCA5	NM_018672	3.50344E-05	1.98902

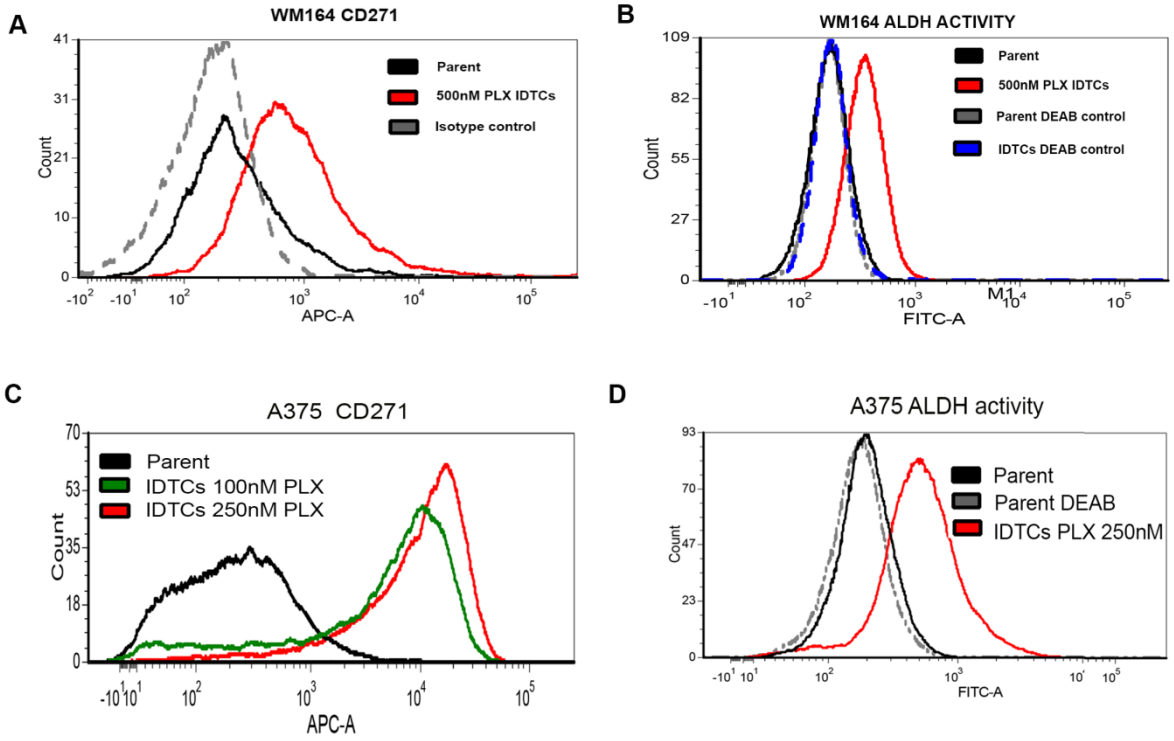


Figure 28: IDTCS show an increased expression of stem cell genes. *(A) Selected stem cell genes from microarray analyses comparing the Log 2 transformed ratio of WM164 IDTCs (Blue) to that of the parent population (green). The mean value of parent samples was equated to zero, and each dot represents one of the triplicates. (B) Table showing the genes represented in (A), their transcript ID, Gene Symbol REF SEQ ID, p- value and fold change.*

Since the expression profile suggested an induction of a stem-like state I reconfirmed the induction of the melanoma stem cell marker CD271 along with ALDH activity which has been widely reported to be a marker for stem cells in cancer and normal tissue (Croker and Allan, 2012; Kim et al., 2013; Marcato et al., 2011b) in WM164 and A375 IDTCs by flow cytometry (Figure 29 A,B,C and D)

The results showed a similar induction of CD271 expression and ALDH activity in both cell lines. Because the in vitro results suggested an induction of stem cell markers we further tested whether the same might happen *in vivo*. To test this we performed experiments in collaboration with Dr. Meenhard Herlyn from The Wistar Institute, Philadelphia.NOD SCID IL2 receptor gamma chain knockout (NSG) mice were used for this experiment to study the induction of tumors using the BRAF mutant 451Lu human melanoma cell line which originates form the WM164 cell line selected for their capacity to spontaneously metastasise to the lungs. After the tumors were found palpable they were treated with a low dose of PLX4720 30mg/kg/day, an altered formulation of PLX4032 effective in mice, for a period of 2 weeks.

We observed no significant reduction in tumour volume at that dosage. This suggests no significant selection pressure. Tumours were harvested after two weeks and tumour tissue embedded in paraffin. Paraffin slides were then subjected to immunohistochemistry and stained for CD271 expression with the help of our collaborators ate the Institute of Pathology, Medical University of Graz. A significant increase in CD271 positive staining was found.

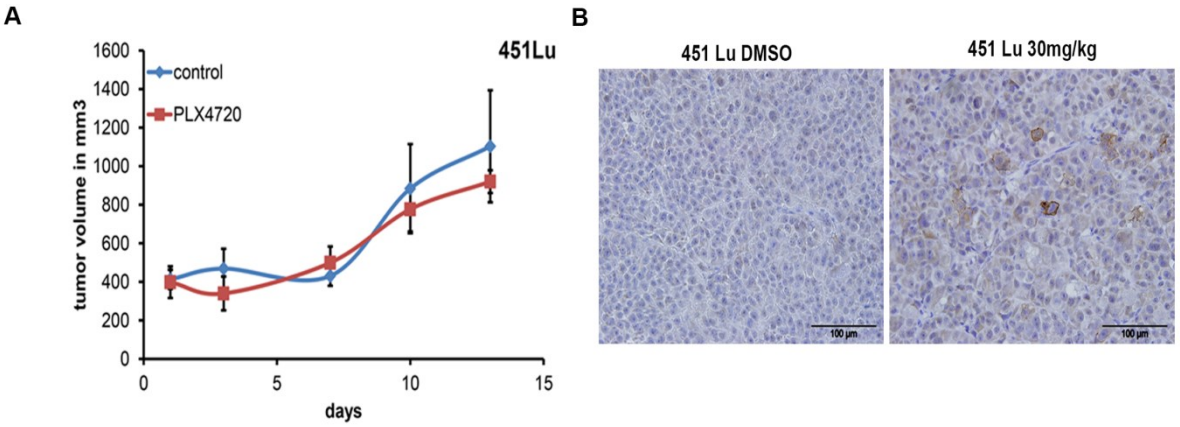


Figure 30: CD271 induction in vivo: *(A) 451 Lu cell- (Wm164 cells propagated in the mouse and isolated from metastases to the lungts) induced tumours were treated for 2 weeks once palpable with 30mg/kg /day PLX4032 or vehicle. Tumors were measured twice weekly using calipers. Error bars represent the standard deviation from the mean. (B) After 2 weeks tumors were harvested and analysed for CD271 expression by immunohistochemistry. (In collaboration with Dr. Clemens Krepler, The Wistar Institute, Immunohistochemistry in collaboration with Silvia Schauer from the Institute of Pathology, Medical University of Graz)*

Since the stem cell like characters would mean a loss of differentiated state we also analyzed the prominent Melanoma differentiation markers melan A (Chen et al., 1996) and tyrosinase (Kwon, 1993) and observed a loss of these markers in IDTC state.

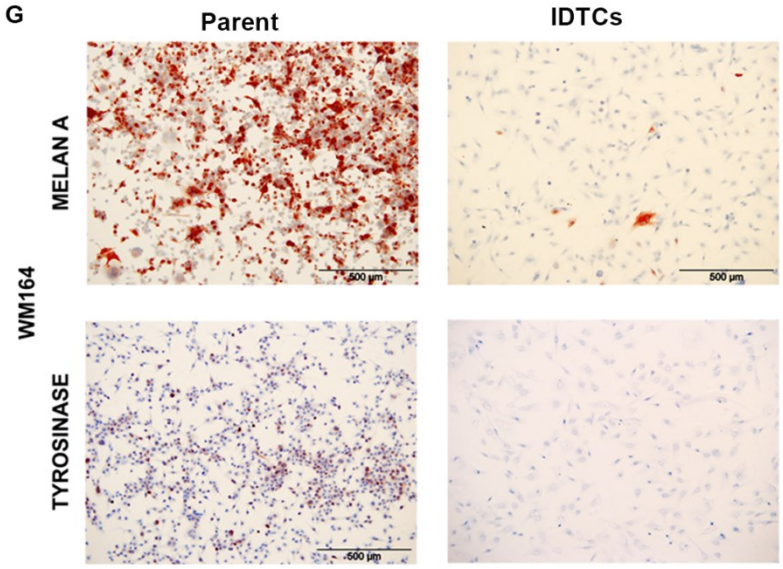


Figure 31: IDTCs lack differentiation marker: *Expression of Melan A and tyrosinase was analysed on WM164 Parent and IDTC by immunocytochemistry. (Done by Department of pathology)*

Further to scrutinize the role of chromatin mediated drug resistance as previously described by Sharma et al chromatin modifying genes based on the microarray data was also analyzed.

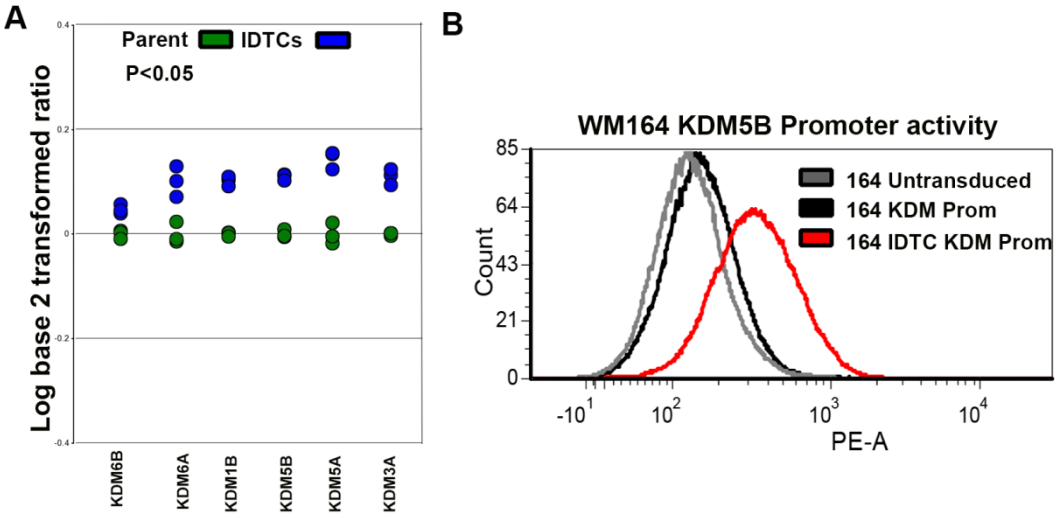


Figure 32: IDTCs show increased expression of chromatin modifying genes. (A) *Selected histone demethylating genes from microarray analysis comparing the Log 2 transformed ratio of WM164 IDTCs (Blue) to that of the parent population (Green) where the mean value of parent samples was equated to zero, and each dot represents one of the triplicates. (B) WM164 IDTCs cells were transduced with a KDM5B DS?s Red promoter construct (Red) and parent cells with a KDM5B promoter construct (Black) to measure promoter activity in the parent state (Black) and IDTC state (Red) by flow cytometry.Microarrays performed by Core facility Molecular Biology Medical university of Graz (Credits to Karin Wagner))*

Sharma et al. had suggested a loss of H3K4me3 as a reason for drug resistance. Therefore I particularly determined the expression of chromatin demethylating proteins and observed an up regulation of genes linked to H3K4me3, including KDM5A, KDM5B and KDM1B, which partly suggests a transcriptional shutdown, as well as an increase in demethylases of H3K27, KDM6A and KDM6B (Figure 32A). Since KDM5B has been reported to be a more important marker in melanoma for a slow cycling drug resistant population, I reconfirmed the expression of this marker by flow cytometry using a KDM5B promoter construct tagged to DS red and transduced WM164 melanoma cells. WM164IDTCs carrying the DS red construct showed a high level of expression of this marker when compared to the parent transduced cells. Untransduced WM164 cells were used a negative control (Figure 32 B). Analyses of WM164 IDTCs for H3K4 and H3K27 methylation showed a down regulation of H3K4me3 and H3K27me3 in IDTCs, whereas there was an increase in H3K9me3 (Figure 33).

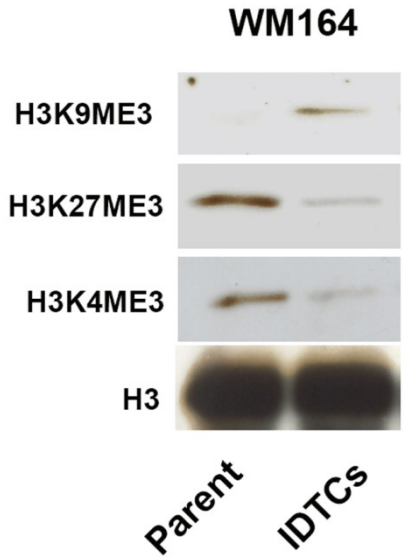
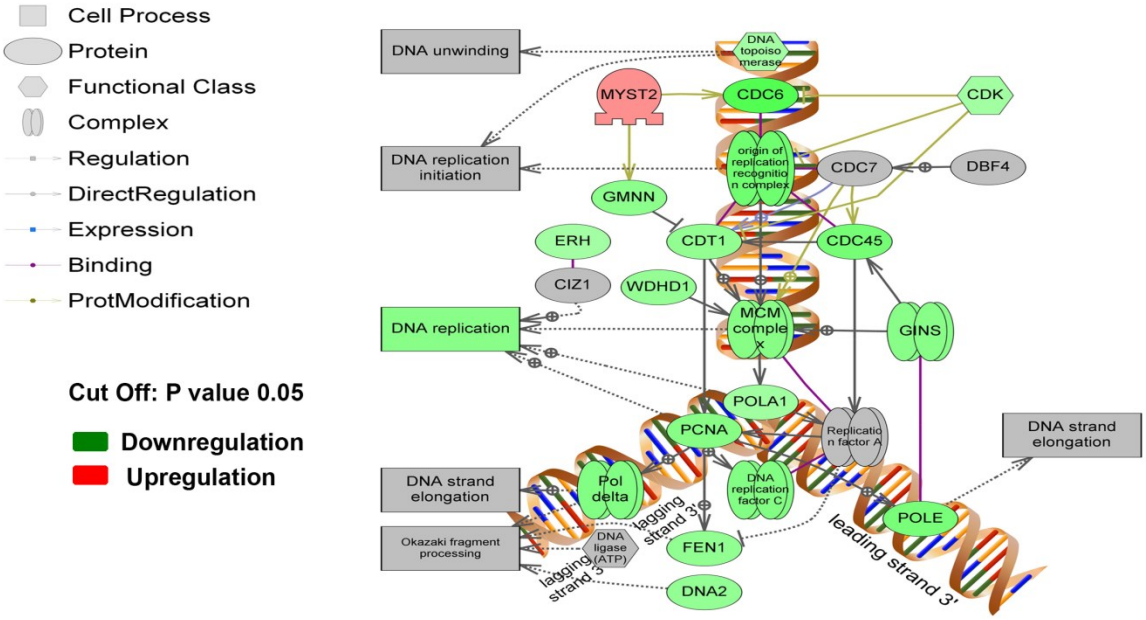


Figure 33: Chromatin remodelling in WM164 IDTC: *Total histone was isolated from parent and WM164 IDTCs and subjected to immunoblotting with H3, H3k4me3, H3K9 me3, and H3K27 me3 antibodies*

Accordingly pathway analysis of microarray data revealed a shutdown of transcription and replication pathways in the IDTC population (Figure 34 A, B) suggesting IDTCs to be in a semi- quiescent state.

Parent VS IDTC



Parent VS IDTC

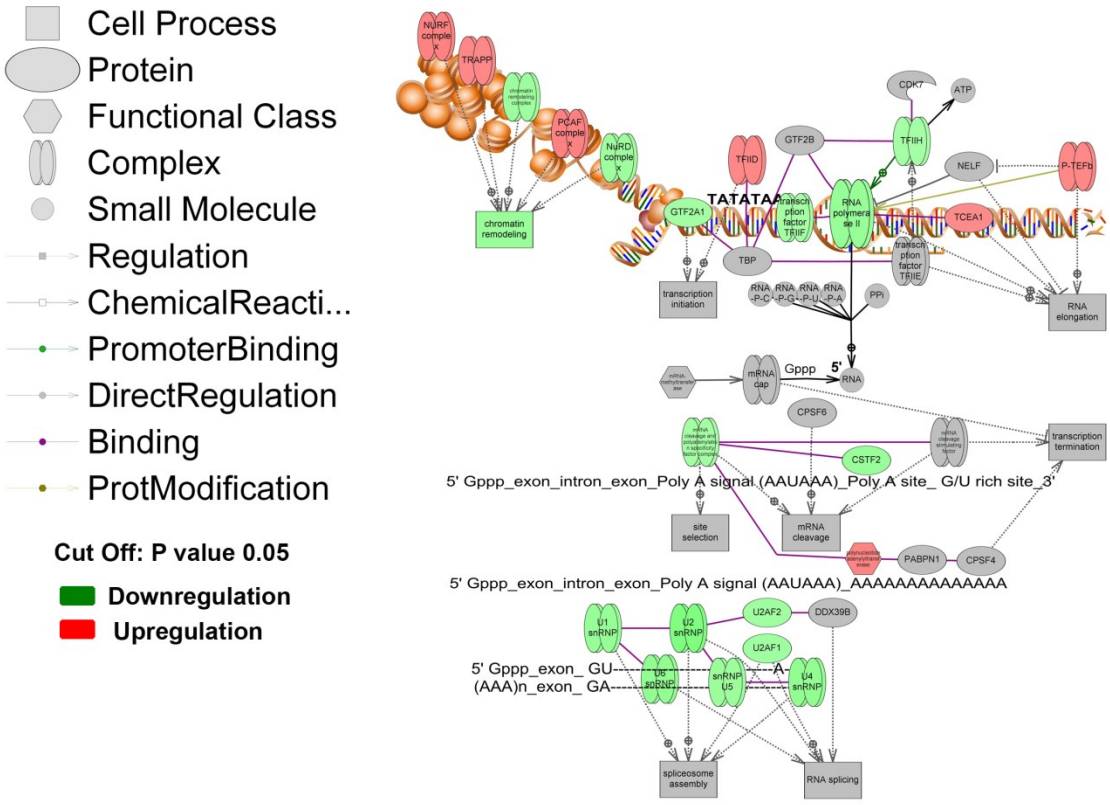


Figure 34: Pathway analyses suggesting a transcriptional and translational shutdown. *Ariadne pathway analysis software showing expression levels of proteins and enzymes involved DNA replication (A) and Transcription (B) in IDTC mRNA when compared to the parent mRNA from the microarray data. Green represents a down regulation of the corresponding mRNA in IDTC and red represents an up regulation. Cut off p value 0.05.*

Altogether the results point to IDTCs mimicking characteristics of both drug resistant stem cells and chromatin mediated drug resistant cells which provide them with drug tolerant capabilities. Although this is in line with previously reported models our results suggest the occurrence of them arising out of the drug exposure itself as an intriguing phenomenon.

(2.2.2) Exposure to persistent stress inducing factors like hypoxia or nutrient starvation generates IDTC-like cells

The formation of IDTCs based on drug exposure together with their characteristic expression profile made me ponder whether environmental factors usually present in the tumor microenvironment like hypoxia or nutrient starvation might also instigate the formation of drug tolerant cells. Because it seemed that generation of IDTCs could be a natural response to unfavorable environmental conditions. Hence we exposed the WM164 cells to 5% O_2 or 1% O_2 hypoxic conditions for a period of 12 days and observed that 1% O_2 led to significant cell death (survival percentage 36.5(+/- 3.74%) equivalent to high PLX4032 doses, whereas at 5% O_2 no significant selection pressure was observed leading to the survival of 122.5% (+/-9.1%) of cells (Figure 35 A). Interestingly and similar to what I found in the case of exposure to PLX4032,, cells exposed to 5% O_2, when further exposed, showed a tolerance towards 1% O_2, suggesting a common capability of cancer cells to cope with extreme conditions. Moreover the experiments were repeated under glucose starving conditions whereupon WM164 cells were subjected to low glucose (1mg/ml: normal is 5mg/ml) and no glucose for a period of 12 days. Without glucose the entire parent population was exterminated in 12 days period whereas at the low glucose condition 95.15% (+/- 1.9 of the cells survived. Again, cells surviving low glucose conditions displayed tolerance towards no glucose when exposed over an additional period of 12 days (Figure 35 B).

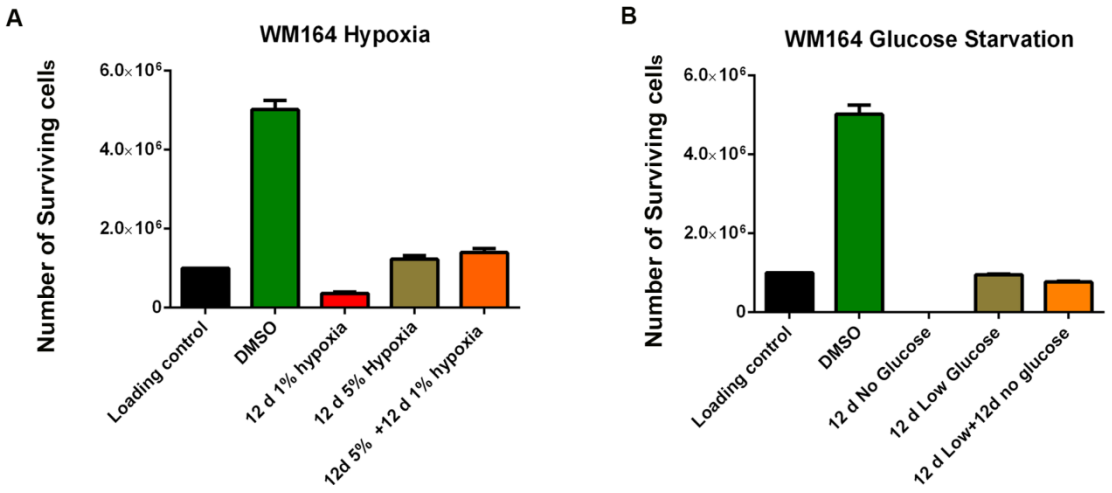


***Figure 35: Hypoxia and glucose starvation leads to an IDTC like phenotype**. (A) 1x10^6 WM164 cells (in duplicate per time point per condition) were subjected to 5% or 1% hypoxic conditions for a period of 12 days and the number of viable cells were counted. Further a 3rd set of cells were treated for 12 days with 5% hypoxia and further with 1 % hypoxia for another 12 days and the number of viable cells were counted and represented as bar graphs. The error bars represent the standard deviation from the mean. (B) The same experimental frame work was repeated whereupon hypoxic conditions were replaced with low glucose conditions. WM164 cells were exposed to no glucose or low glucose (1mg/ml) for a period of 12 days and the number of viable cells was counted. Further the low glucose treated cells from an experiment in parallel were subjected to no glucose media for another 12 days and the number of viable cells was counted and was plotted as a graph. The error bars represent the standard deviation from the mean.*

Further IDTCs generated out of low glucose and 5% O_2 showed an increase in CD271 expression and elevated ALDH activity in both populations (Figure 36).

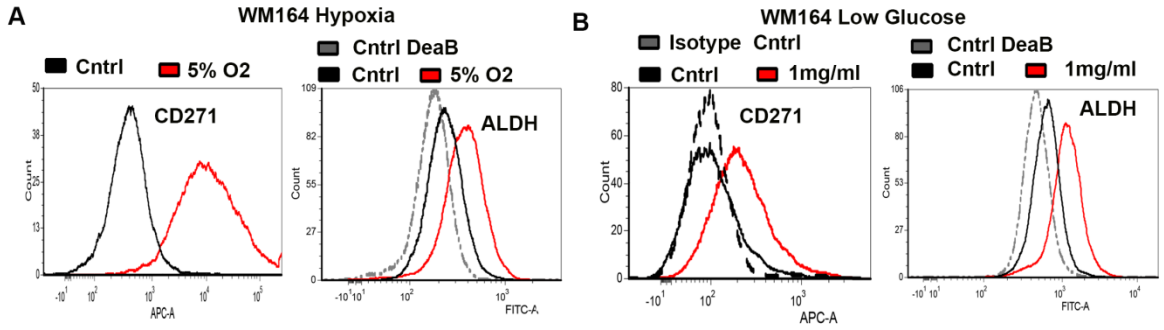


***Figure 36: Hypoxia and low glucose exposed cells show increased expression of CD271 and ALDH activity in WM164 cells**. WM164 parent cells, cells exposed to 5% hypoxia(A) or Low glucose (B) for 12 days, were analysed for their CD271 and ALDH activity by facs.*

The experiment of exposure to hypoxic conditions was repeated in A375 cells to rule out a cell line specific effect and the results were similar (Figure 37).

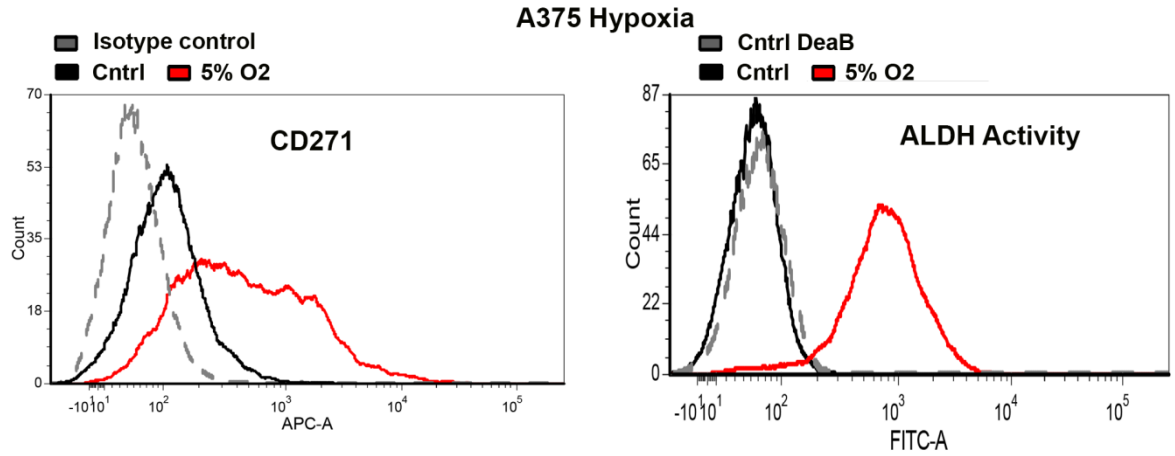


Figure 37: A375 cells exposed to hypoxia show increased expression of CD271 and ALDH activity. *(Left) CD271 expression of A375 parent (Black) and 5% hypoxia IDTCs (Red) subjected to flow cytometry. (Right) ALDH activity of A375 parent (black) and 5% hypoxia IDTCs (red) compared to their respective DEAB negative control.*

Additionally, hypoxia and low glucose generated IDTCs were probed by RT-PCR for the expression of distinct genes that were upregulated in the microarray analyses from PLX4032 experiments. Indeed an increase in ABCB5, the stem cell marker OCT4 and KDM5A expression under both conditions was observed (Figure 38).

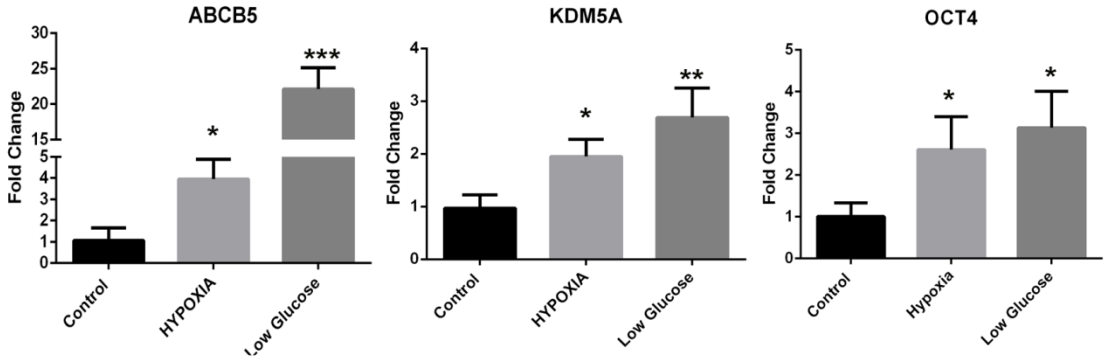


Figure 38: ***WM164 cells exposed to hypoxia and low glucose show increased expression of ABCB5, KDM5A and OCT4 by RT-PCR.*** *mRNA isolated from WM164 parent cells exposed to 5% hypoxia and low glucose, for 12 days were analysed for their ABCB5, KDM5A and OCT4 expression. Error bars represent the standard deviation from the mean. Statistical analysis was done using the T test by comparing each treatment against the parent cells, P value is represented by (*) where P</= 0.0001 is represented by (****) , P</= 0.001 by(***), P</= 0.01 by (**) and P</= 0.05 by(*)*

The expression of certain markers investigated seems to be context specific. For example the expression of KDM5B was only observed under hypoxic conditions and SOX10 only in cells exposed to low glucose (Figure 39).

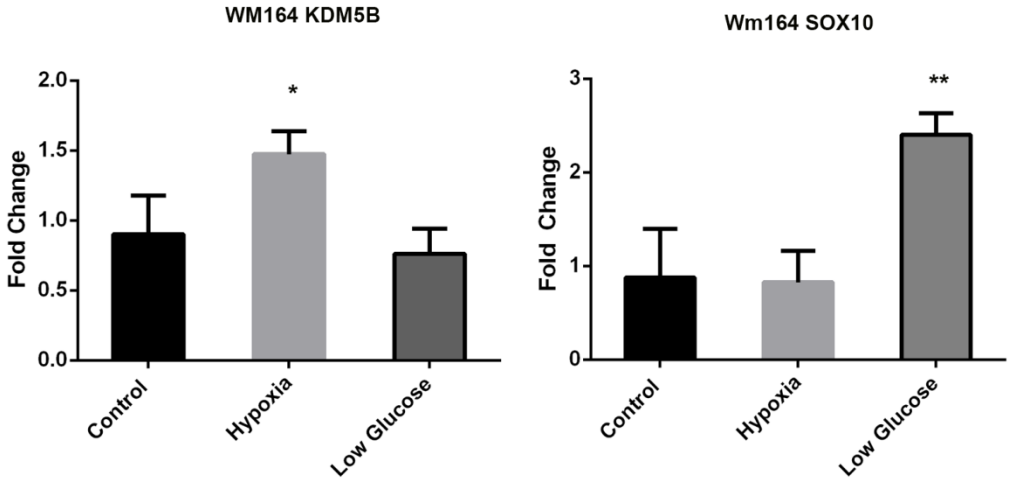


Figure 39 : KDM5B and SOX10 show context specific expression*. mRNA isolated from WM164 parent cells exposed to 5% hypoxia and low glucose for 12 days were analysed for their KDM5B and SOX10 expression by RT –PCR. Error bars represent the standard deviation from the mean. Statistical analysis was done using the T test by comparing each treatment against parent cells, P value is represented by (*) where P</= 0.0001 is represented by (****) , P</= 0.001 by(***),, P</= 0.01 by (**) and P</= 0.05 by(*)*

However both, hypoxia and low glucose, generated cell types with increased ERK and AKT phosphorylation (Figure 40A) along with the loss of H3K4me3 and H3K27me3 and a gain of H3K9me3 (Figure 40B). The increase in ERK phosphorylation in IDTCs resulting from BRAF inhibitor treatment was thought to be a drug specific response of the cells to the BRAF inhibitors bypassing BRAF signaling and thereby resistance to the drug, as previously reported (Poulikakos and Rosen, 2011). Surprisingly and unexpectedly, this pattern appears to be the same in cells arising from hypoxic and low glucose exposure suggesting a generic response contributing to ERK activation.

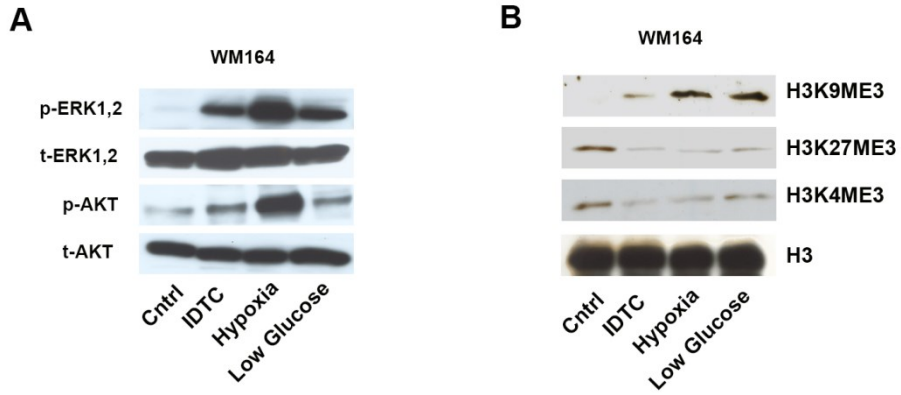


Figure 40: WM164 cells exposed to low glucose and hypoxia show an increase in AKT and ERK signalling along with an altered chromatin state. *(A)Lysates of WM164 500 IDTCs, parent cells and cells exposed to 12 day hypoxia or low glucose were subjected to immunoblotting to detect phosphorylated ERK1,2 (p-ERK1,2(Thr202/Tyr204)) , total ERK(T-E RK1,2)), phosphorylated AKT (p-AKT (Ser473)) and total AKT (T-AKT). (B) Total histone was isolated from parent, WM164 IDTCs and cells exposed to 12 days of hypoxia or low glucose, and subjected to immunoblotting with H3, H3K4me3, H3K9 me3, and H3K27 me3 antibodies.*

Altogether, if the marker expression of IDTCs cells evolving under hypoxic or low glucose conditions were compared a high similarity was found. Thus I further probed whether these cells exhibit multiple drug tolerance by exposing them to PLX4032, GSK1120212 and cisplatin (Figure 41A).

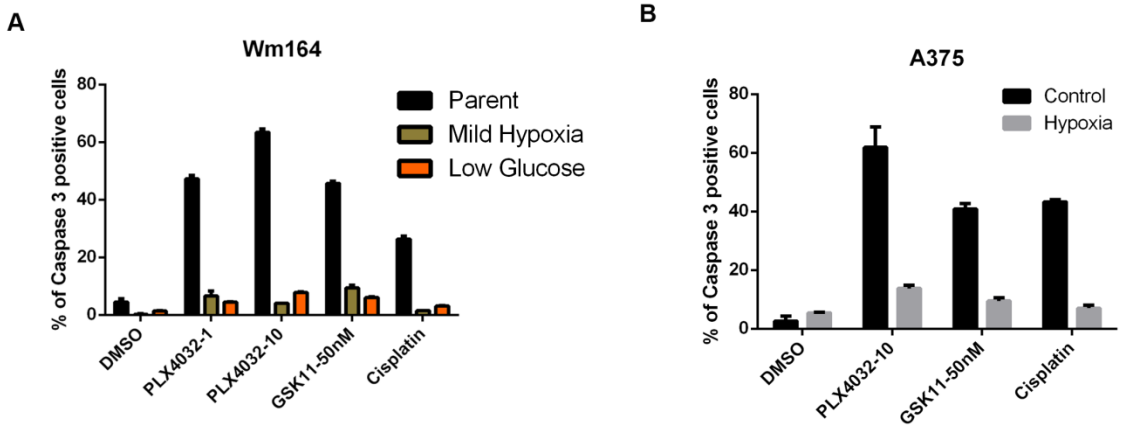


Figure 41: WM164 cells exposed to low glucose and hypoxia show multiple drug tolerance. *(A)WM164 cells exposed to 12 days of hypoxia or low glucose were challenged with PLX4032 at 1 μM, 10μM, GSK1120212 50nM and cisplatin 10μM for 48 hours and the percentage of caspase 3 positive cells was analysed by flow cytometry and compared to the parent population. Error bars represent the standard deviation from the mean. (B) A375 cells exposed to 12 days of hypoxia were challenged with PLX4032 at 10μM, GSK1120212 50nM and cisplatin 10μM for 48 hours and the percentage of caspase 3 positive cells were analysed by flow cytometry and compared to the parent population. Error bars represent the standard deviation from the mean.*

A low caspase 3 activity was found for cells under hypoxic and low glucose conditions if compared to parent cells indicating tolerance towards the different drugs. Similar results were obtained in A375 cells exposed to 5%O2 as well (Figure 41 B). These data suggest that an IDTC like state can be driven by common micro-environmental factors like hypoxia or low nutrient availability, which point to a general early innate response to persistent hazardous conditions. The conversion harnesses these cells to tolerate diverse strident environments.

(2.2.3) IDTCs display functional characteristics of cancer stemness

Since IDTCs show multiple stem cell-like signatures I determined whether they deliver certain features of stemness. One mainstay for testing of stem cell like characteristics is the sphere formation assay. The non-adherent sphere forming capability of cancer stem cells has been reported in multiple cancer types including melanoma (Fang et al., 2005). Hence, parent cells and IDTCs were plated at 50, 500 and 5000 cells in ultra-low attachment plates for a period of 7 days in serum free media supplemented with 10ng/ml of FGF and EGF to examine for sphere forming capacity. I observed that IDTCs are highly capable of forming melanoma spheres when compared to the parent cells even at 50 cells, for which parent cells failed to initiate the formation of melanoma spheres (Figure 42). A significantly higher sphere

forming capability in melanoma IDTCs was also observed at higher cell numbers.

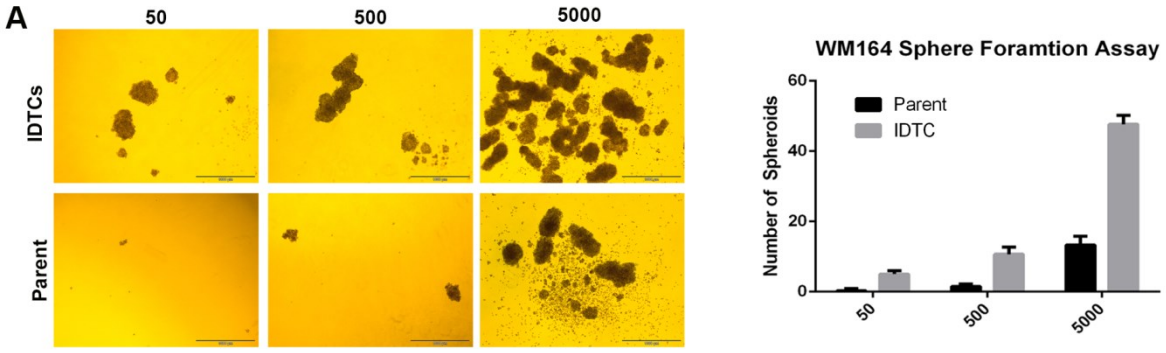


Figure 42: IDTCs have higher sphere forming capability. *Representative pictures of WM164 parent and 500nM IDTC sphere formation when plated at 50, 500, 5000 cell density per well during a 7 days period (left). Experiments were done in triplicate and the number of spheres per well was quantified and represented as bar graphs (right).*

The tumor initiating capability of cancer cells is another standard experiment to test their stem like characteristics. To this end and based on the above presented results I tested the tumorigenic potential of IDTCs *in vivo* using a xenograft NOD.CB17-Prkdcscid/J mouse model by injecting 50, 500 and 5000 cells each of either WM164 IDTCS or parent cells. I observed palpable tumors at the IDTCs injected sites within 20 days for both the 500 and the 5000 group whereas tumor development was delayed by more than a week at the sites where parent cells were injected. Tumor volume from parent cells was also found to be significantly lower than IDTC tumors. This phenomenon was even more strikingly observable in the 50 cells group, matching our *in vitro* observation, whereupon the delay in palpable tumor formation was over 2 weeks and the tumor volume showed an over ten times difference (Figure 43).

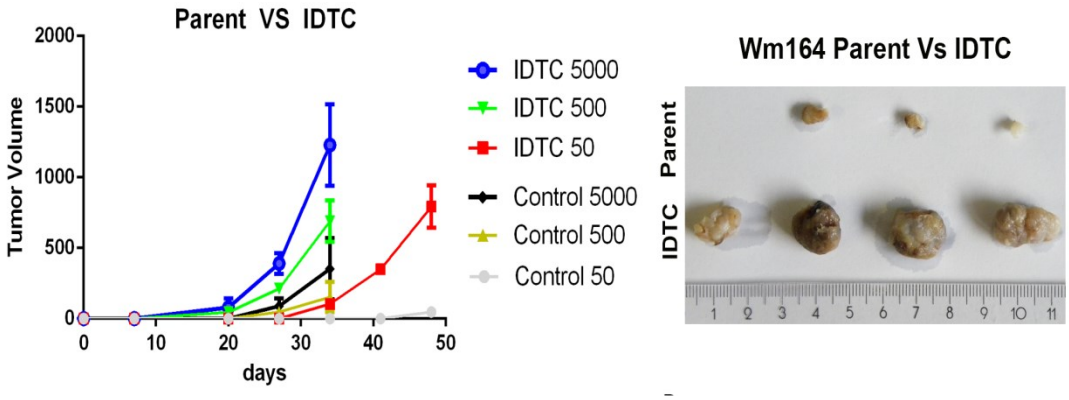


Figure 43: IDTCs have a higher tumourigenic potential in SCID mice. *Tumour volume after subcutaneous injection into NOD.CB17-Prkdcscid/J mice of WM164 parent or 500nM IDTCs (15 days treatment) at 50(n=4), 500(n=4) or 5000(n=5) cells measured over the entire time period of the experiment (Left). (Right)Rrepresentative pictures of WM164 parent and IDTC tumors in the 50 cells group at the end of experiment.*

Altogether our results suggest that IDTCs mimic a highly tumorigenic stem cell like behavior similar to what has been demonstrated for CD271+ve melanoma initiating cells (Boiko et al., 2011; Civenni et al., 2011).

Another hallmark of cancer cell plasticity is their capacity to express angiogenic factors and thereby initiating the formation of vessel-like structures, which has been coined vasculogenic mimicry (Fan et al., 2013; Hendrix et al., 2003). The high tumorigenic capacity of IDTCs prompted us to test whether they exhibit defined signatures of angiogenesis. On testing I observed a subpopulation of CD271+ve IDTCs showing high levels of CD31 expression which was very low in the parent cell population (Figure 44). WM164 IDTC spheroids showed an even increased number of CD271 and CD31 double positive cells if compared to parent cells grown to spheroids (Figure 44).

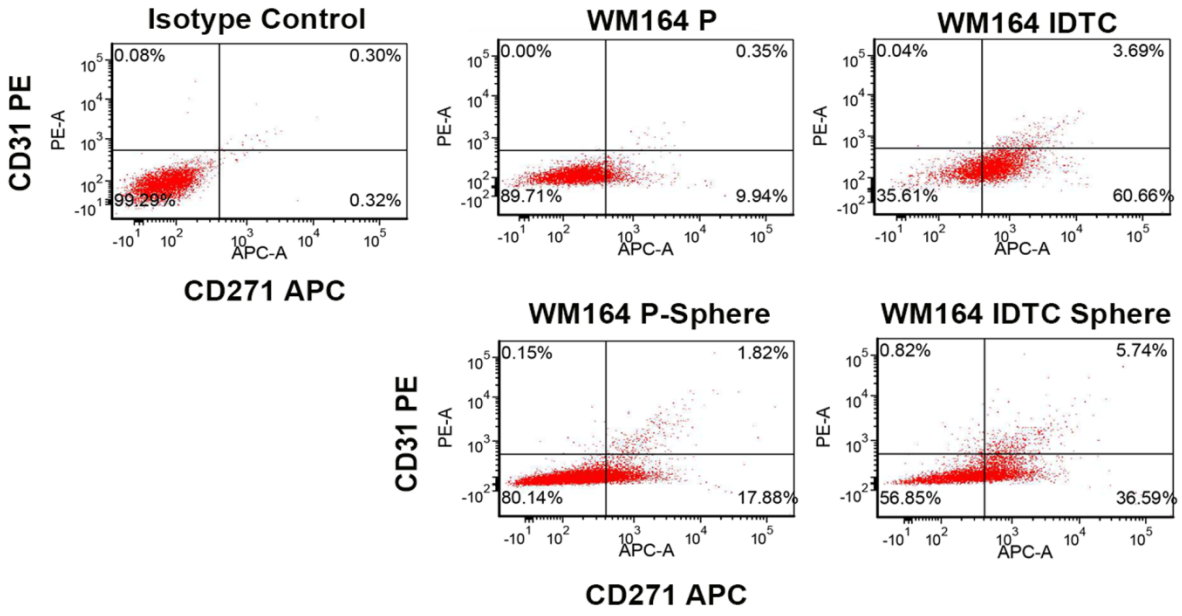


Figure 44 : IDTCs display with a subpopulation of cells which are CD31+ve.*WM164 parent and IDTCs (top) were facs analysed by flow cytometry for their CD271 and CD31 expression, (bottom) WM164 parent and IDTCs allowed to develop into spheres were reanalysed for their CD271 and CD31 expression.*

Further I tested the expression of multiple angiogenic secretory molecules in the parent and IDTC conditioned media using an angiogenic array.IDTCs secreted high levels of multiple angiogenic factors like angiogenin, angiopoietin1, angiopoietin2, artemin, EGF, VG-VEGF, HGF, and IGFBP2 compared ot parent cells (Figure 45).

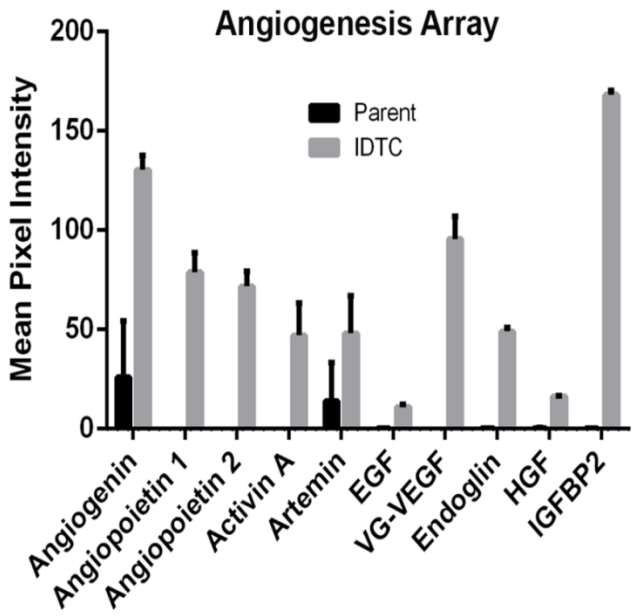


Figure 45: IDTCs secrete angiogenic cytokines and growth factors*. Mean pixel intensity of secreted angiogenic cytokine and growth factors of WM164 parent and IDTCs from angiogenesis array analysed by Image J background subtraction method and plotted as bar graphs. Error bars represent the standard deviation from the mean. Statistical analysis was done using the T test and P values are represented by (*) where P</= 0.0001 is represented by (****) , P</= 0.001 by(***),, P</= 0.01 by (**) and P</= 0.05 by(*).*

Correspondingly, IDTCs exhibited profound increased endothelial cell invasion properties compared to parent cells, which is reported to be crucial for exerting angiogenic capabilities(Lamalice et al., 2007)(Figure 46 A).

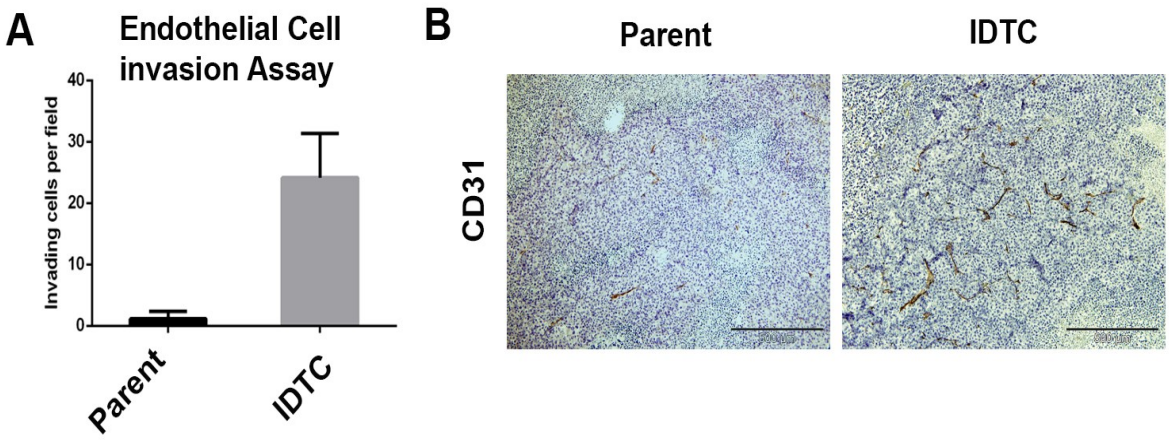


Figure 46 : IDTCs show enhanced endothelial migration and vessel forming capacities. *Endothelial cell invasion assay showing the mean number of invading cells per 10X field from experiments done in triplicates and two different fields being analysed from each experiment. Error bars represent the standard deviation from the mean. (F) CD31 expression from WM164 IDTC tumors (500 group) and parent tumors (500 group) by subjecting fixed tissue to immunohistochemistry analysis. (Immunohistochemistry done by pathology department, medical University Graz(Credits to Sylvia Schauer))*

Our *in vitro* results were also corroborated by staining tumour tissue of the xenografts with CD31 for analyzing vessel formation. IDTC tumours showed increased vessel formation compared to parent tumours (Figure 46 B)

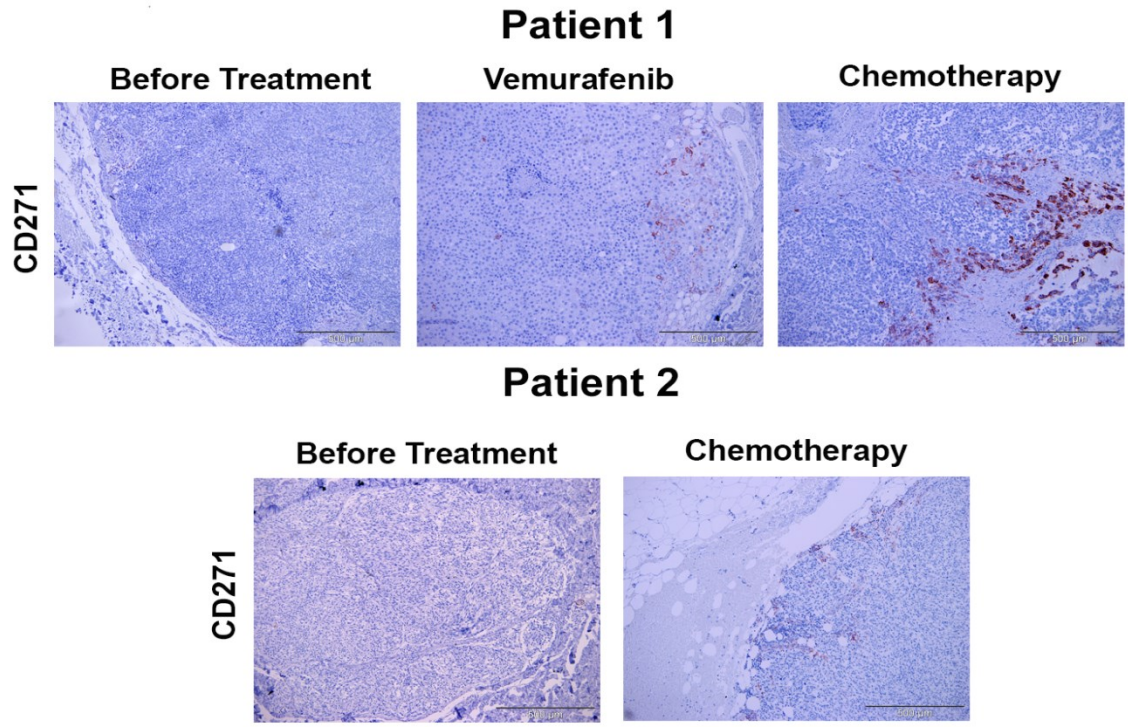


Figure 47: CD271 cells colocalizes with tumor vessels. *(Patient 1) Tissue slides prepared from biopsies of subcutaneous metastases of a melanoma patient stained for CD271 before treatment, after 8 months of treatment with Vemurafenib (Zelboraf®) and after 2 months of treatment with taxol and cisplatin. (Patient 2) Biopsies from subcutaneous metastases of a patient, before treatment (left) in comparison to one month after the start of treatment with dacarbazine (right)*

We tested supporting proof for the same in two matched patient samples before and after therapy, where we could observe an increased number of CD271+ve cells after treatment. What was more interesting to note is that the majority of CD271+ve cells were located in close proximity to the blood vessels in the tumour (Figure 47). Together our studies indicate that the transition of parent cells into IDTCs goes along with an increased angiogenic potential which might explain the phenomenon of increased tumorigenicity.

(2.2.4) CD271 or KDM5B knockdown increases the susceptibility to various drugs but the emerging IDTCs still exhibit multiple drug tolerance

The generation of IDTCs as an early response to drug treatment pose a serious hindrance for successful trade-offs of various treatments by not only exerting multiple drug tolerance but also by evolving to a population of cells with a highly tumorigenic

potential. Therefore targeting this population is of high priority. Previous reports have suggested combination strategies of primary drug with IGF1R inhibitor , the AKT pathway inhibitor and the HDAC inhibitor, could eradicated a pre-existing slow cycling multiple drug tolerant population (Sharma et al., 2010). I tested these strategies with OSI906 (IGF1R inhibitor), LY294002 (Pi3K/AKT inhibitor), TSA (HDAC inhibitor) on the IDTCs arising from PLX4032 treatment as well as that of 5% hypoxia and low glucose. Independent of the combination and the source of IDTCs increased cell death were always observed in the parent population (Figure 48).

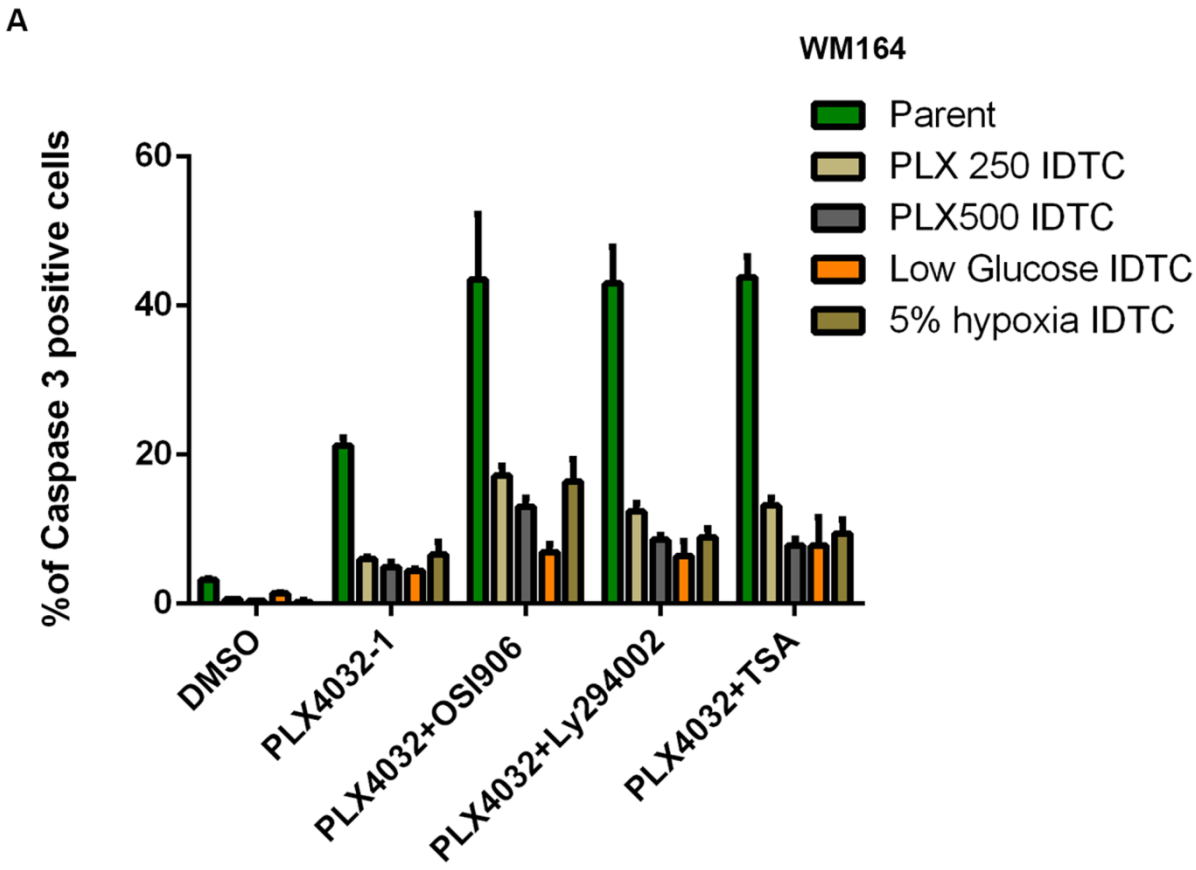


Figure 48: IDTCs are tolerant towards strategies that eradicate slow cycling cells: *WM164 Parent, PLX4032 250nM IDTCs (PLX 250 IDTC), PLX4032 500 nM IDTCs (PLX 500 IDTC), Low glucose exposed IDTCs (Low glucose IDTC) and 5%O_2 hypoxia induced IDTCs (5% hypoxia IDTC) were exposed to either DMSO or PLX4032 alone (1μM) or PLX4032 (1μM) in combination with one of the following inhibitors: OSI906 (5 μM), Ly294002 (10 μM), TSA (100nM) for a period of 36 hrs and active caspase 3 expression in cells was analysed by flow cytometry and plotted as bar graphs. Error bars represent the standard deviation from the mean.*

Even exposure over 12 days to these drug combinations of already established IDTCs did not result in a notable effect on their survival rather the combination seems to induce a minor slowdown in their growth rate if compared to PLX4032 alone(Figure 49).

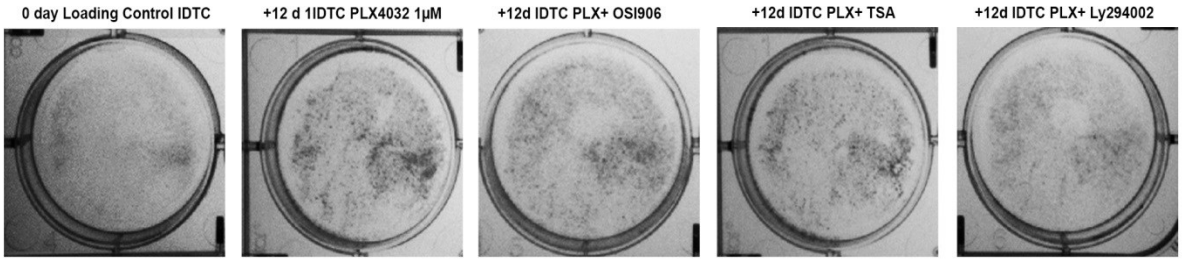


Figure 49: IDTCs show a decreased proliferation if exposed to drug combinations. *Representative pictures of 1x10^5 WM164 IDTCs plated per well and exposed over a period of 12 days to 1μM PLX4032 alone or in combination with OSI906 (5 μM), Ly294002 (10 μM), or TSA (100nM) and the surviving cells were fixed and stained with crystal violet.*

The results prompted me to look for alternative approaches. The increased CD271 expression as a characteristic marker of IDTCs suggested that by silencing it IDTCs might be re-sensitized similar to what has been reported for KDM5B in eliminating a slow cycling melanoma population (Roesch et al. 2012).HenceI developed WM164 cells constitutively expressing CD271 and KDM5B sh RNAs using lentiviral particles. The cells were further selected with puromycin for over a week to ensure selection of cells stably expressing sh RNA constructs. Indeed stable knockdown of either CD271 or KDM5B in the parent cells significantly increased caspase 3 activity compared to control sh RNAs exposed to single or combined drugs (Figure 50).

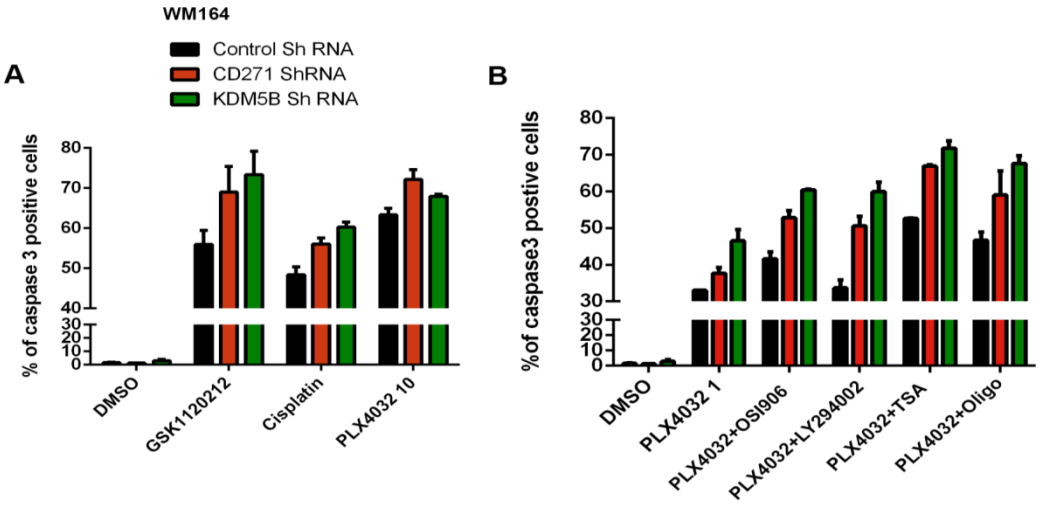


Figure 50: WM164 shCD271 and shKDM5B cells show increased susceptibility to drugs. *(A)WM164 control shRNA transduced (Black), shCD271 RNA transduced (Red) and shKDM5B RNA transduced cells (Green) were subjected to treatment with GSK1120212 (50nM), Cisplatin (30 µM) or PLX4032 (10 µM) for 48 hours and the percentage of caspase 3 positive cells were analysed by FACS and plotted as bar graphs., Error bars represent the standard deviation from the mean. (B) WM164 control sh RNA transduced (Black), shCD271 RNA transduced (Red) and shKDM5B RNA transduced (Green) cells were subjected to treatment with PLX4032 (1µM) or in combination with one of the following inhibitors: OSI906 (5 µM), Ly294002 (10 µM), TSA (100nM) or Oligomycin A (1 µg/ml) and the percentage of caspase 3 positive cells plotted as bar graphs. Error bars represens the standard deviation from the mean*

Prolonged exposure over 12 days to different concentrations of PLX4032 also showed similar results, whereupon the number of surviving cells was considerably reduced in shCD271 and shKDM5B transduced cells even at 250nM and 500nM

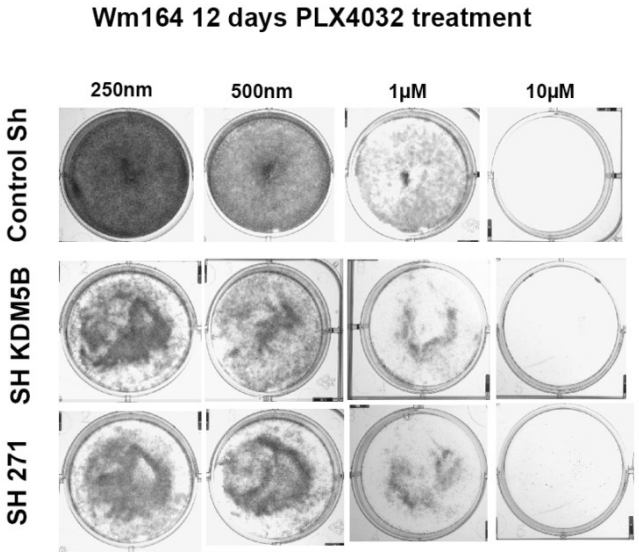


Figure 51: WM164 shCD271 and KDM5B cells are susceptible to even low concentrations of PLX4032: *WM164 control sh RNA transduced, shCD271 RNA transduced and shKDM5B RNA transduced cells were subjected to exposure to to PLX4032 at 250nM, 500nM, 1µM or 10 µM for a period of 12 days and the surviving cells were stained with crystal violet.*

However, the cells that survived the drug exposure for 12 days belonging to both the shCD271 and the shKDM5B populations to our surprise invariably showed drug tolerance nearly equally to that of IDTCs rising from the control shRNA transduced parent cell population(Figure 52).

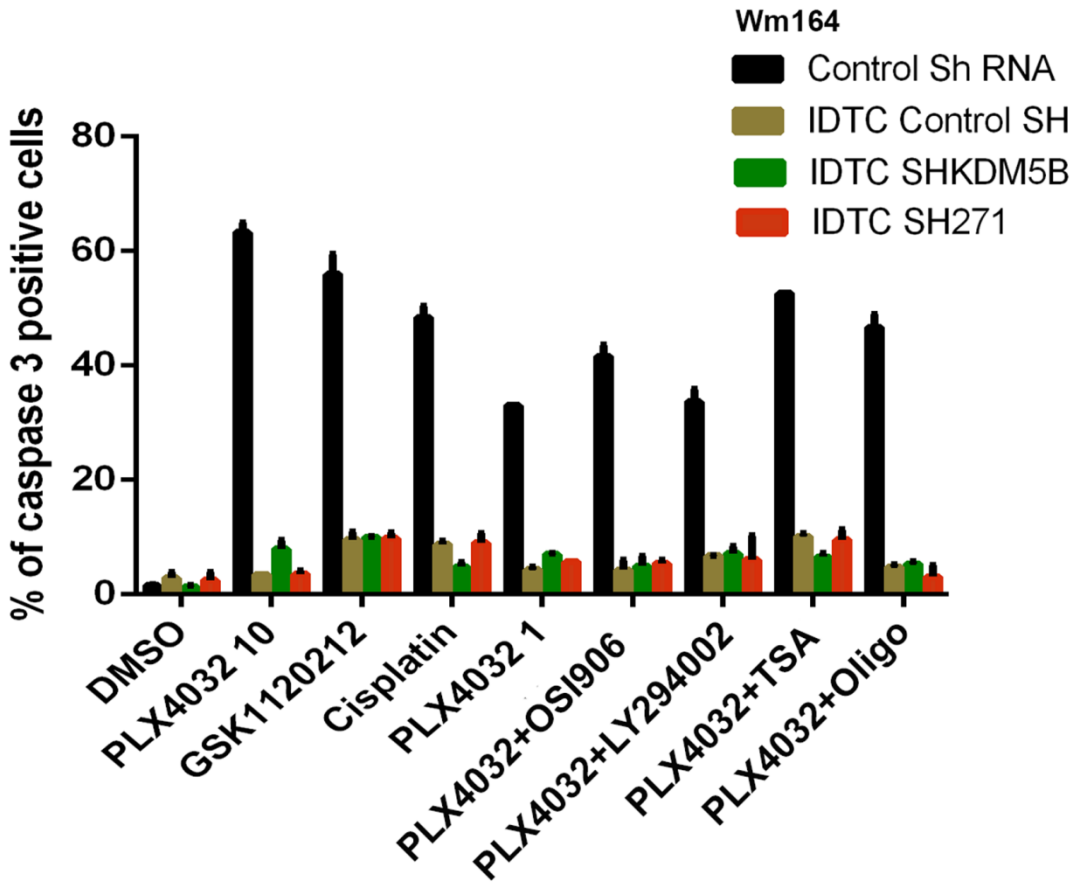


***Figure 52**: **sh CD271 and sh KDM5B IDTCs still exhibit drug tolerance.** WM164 control sh RNA transduced, control sh RANA transduced IDTCs, sh CD271 RNA transduced IDTCs and shKDM5B RNA transduced IDTCs were exposed to GSK1120212 (50nM), Cisplatin (30 µM), PLX4032 (10µM), PLX4032 (1µM) and PLX4032 (1µM) in combination with one of the following inhibitors OSI906 (5µM), Ly294002(10 µM), TSA (100nM) or Oligomycin A (1µg/ml) for 48 hours and the percentage of caspase 3 positive cells were analysed by flow cytometry and plotted as bar graph. Error bars represent the standard deviation from the mean.*

This was even true for a combination with oligomycin A which targets the mitochondrial survival pathways and has been reported to eradicate slow cycling cells (Roesch et al., 2013)

The results suggest that knockdown of CD271 and KDM5B reduced the probability of transition into IDTCs but did not further sensitize the already transformed IDTCs. The expression of CD271 and KDM5B was analyzed in the surviving shCD271 and

shKDM5B IDTCs to ensure that the results observed were not due to inefficient knockdown of protein or loss of sh RNA expression (Figure 53).

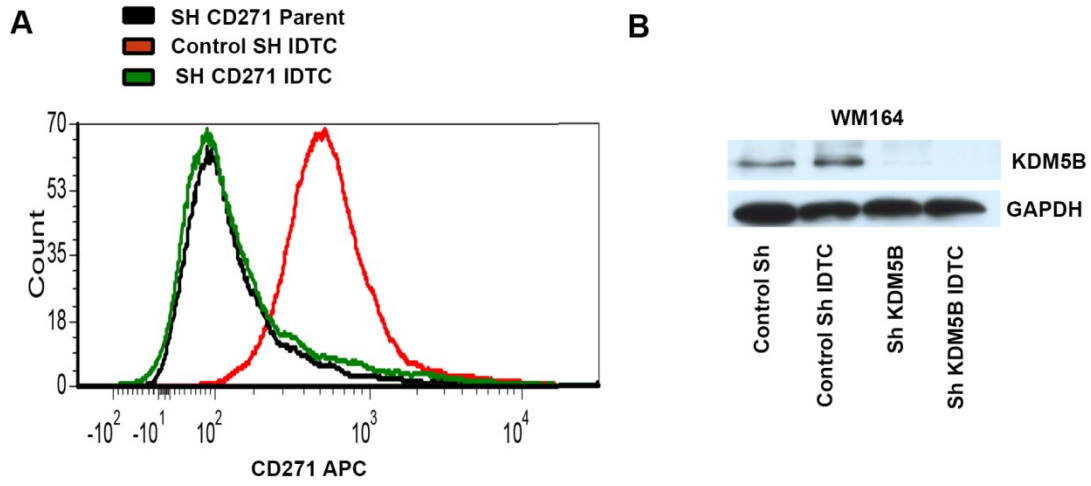


***Figure 53: Confirming the knockdown of CD271 and KDM5B in IDTCs**. (A) WM164 shCD271 RNA transduced parent, shCD271 RNA transduced IDTCs, control shIDTCs subjected to CD271 expression analysis by flow cytometry. (B) Immunoblot analysis of protein lysates from WM164 control shRNA transduced cells (Control sh), Control shRNA transduced IDTCs (Control sh IDTC), shKDM5B RNA transduced cells (sh KDM5B) and shKDM5B transduced IDTCs (sh KDM5B IDTCs).*

(2.2.5) IDTCs activate multiple signalling pathways allowing for desensitizing to targeted inhibitors and rewiring of signalling pathways

Our observation that IDTCs remain insensitive to multiple drug treatment strategies even after targeting the pathways they depend on is intriguing. Because IDTCs showed an increase in multiple drug efflux genes and drug effluxing has been previously described to be an important tool in mediating drug resistance I tried to understand whether drug efflux could be a reason behind their tolerance. To test this I exposed PLX4032 IDTCs to a combination of PLX4032 and GSK1120212 since they showed a high level of MEK phosphorylation. As expected exposure to GSK1120212 completely inhibited MEK phosphorylation at any time points, implying that drug efflux is not leading to the reactivation of MEK signalling (Figure 54 A).

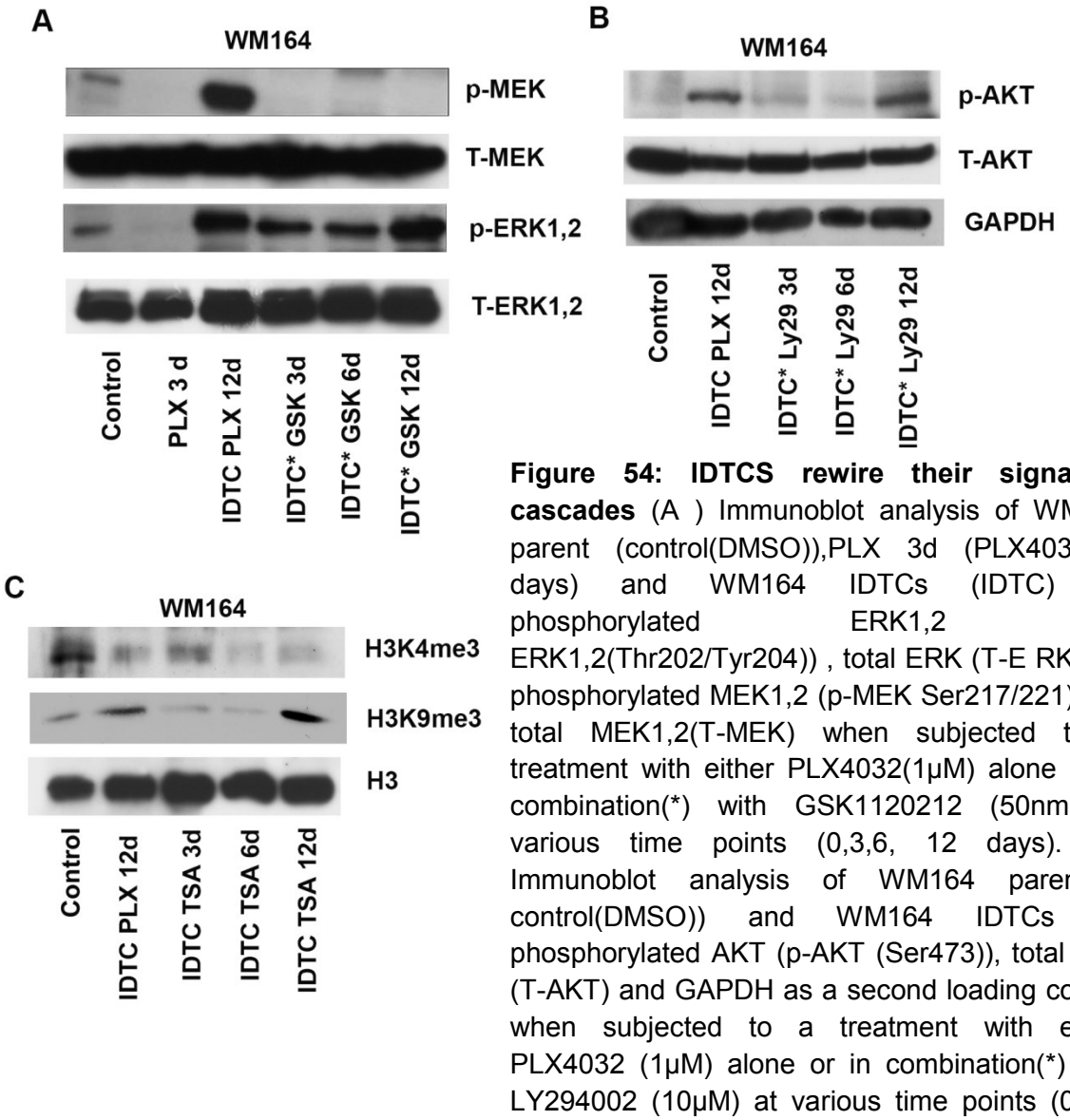


Figure 54: IDTCS rewire their signalling cascades (A) Immunoblot analysis of WM164 parent (control(DMSO)),PLX 3d (PLX4032 3 days) and WM164 IDTCs (IDTC) for phosphorylated ERK1,2 (p-ERK1,2(Thr202/Tyr204)) , total ERK (T-E RK1,2), phosphorylated MEK1,2 (p-MEK Ser217/221) and total MEK1,2(T-MEK) when subjected to a treatment with either PLX4032(1μM) alone or in combination(*) with GSK1120212 (50nm) at various time points (0,3,6, 12 days). (B) Immunoblot analysis of WM164 parent (control(DMSO)) and WM164 IDTCs for phosphorylated AKT (p-AKT (Ser473)), total AKT (T-AKT) and GAPDH as a second loading control when subjected to a treatment with either PLX4032 (1μM) alone or in combination(*) with LY294002 (10μM) at various time points (0,3,6, 12 days).

C) Immunoblot analysis of WM164 parent (control(DMSO)) and WM164 IDTCs for H3, H3k4me3, H3K9 me3 when subjected to a treatment with either PLX4032 (1μM) alone or in combination(*) with TSA (100nM) at various time points (0,3,6, 12 days).

It was interesting to note that ERK phosphorylation was only partially inhibited by GSK1120212 indicating that ERK phosphorylation is not exclusively wired through MEK phosphorylation. Also, GSK1120212 induced downregulation of ERK phosphorylation lasted only for several days as the IDTCs regained ERK phosphorylation to initial levels again within a period of 12 days. This suggests a rewiring of ERK signaling independently of MEK phosphorylation, which explains their insensitivity to the MEK inhibitor (Figure 54 A). The phenomenon of rewiring in IDTCs subsequently resulting in drug tolerance was tested for Pi3k/AKT and HDAC

inhibitors as well. For this purpose, IDTCs were exposed to a combination of PLX4032 along with either the PI3K inhibitor Ly294002 or the HDAC inhibitor TSA for a period of 12 days and cells were harvested for analysis at days 3, 6 and 12. When Ly294002 was used in combination we observed a down regulation of AKT signaling at days 3 and 8, but at day 12 again a reactivation (Figure 54 B). HDAC inhibitors have been reported to positively affect H3K4me3 which has been linked to transcriptional activation of promoters (Koch et al., 2007) and negatively affecting H3K9me3 (Huang et al., 2011) a transcriptional repressor (Barski et al., 2007) which should invoke a more transcriptionally active state in IDTCs. Therefore we probed expression levels of these two markers after exposure of IDTCs to TSA, a HDAC inhibitor at 100nM. At day 3 an increase in H3K4me3 and loss of H3K9me3 was observed whereas during the 12 days treatment period expression levels of H3K4me3 and H3K9me3 reversed to IDTC levels before exposure to TSA (Figure 54C). Taken together these results suggest that IDTCs have a unique capability to rewire through multiple signaling pathways in order to regain their innate IDTC state.

This phenomenon prompted me to look for underlying factors contributing to the rewiring. I suspected several mechansims since the results suggest that targeting the cells through multiple approaches including glucose starvation, hypoxia or individual genes all led to the same outcome. It also suggests that rather than individual genes or molecules a network of signaling cascades could be the underlying cause. Hence we subjected the WM164 parent and IDTC cell lysates to RPPA analyses to understand the underlying signaling pathways contributing to the IDTC state. Compared to the parent cells a significant increase in activation of ATM (pS1981) (Khalil et al., 2011), EGFR (pY1068) (Grant et al., 2002; Saito et al., 2004), FGFR (pY653) (Mohammadi et al., 1996; Zou et al., 2012), YAP (pS127) (Overholtzer et al., 2006), FAK (pY397) (Glover et al., 2004) and SRC (pY416) (Kim et al., 2009a) was found in IDTCs, all of which have been reported to be potential activators of both ERK and AKT signaling (Figure 55 A).

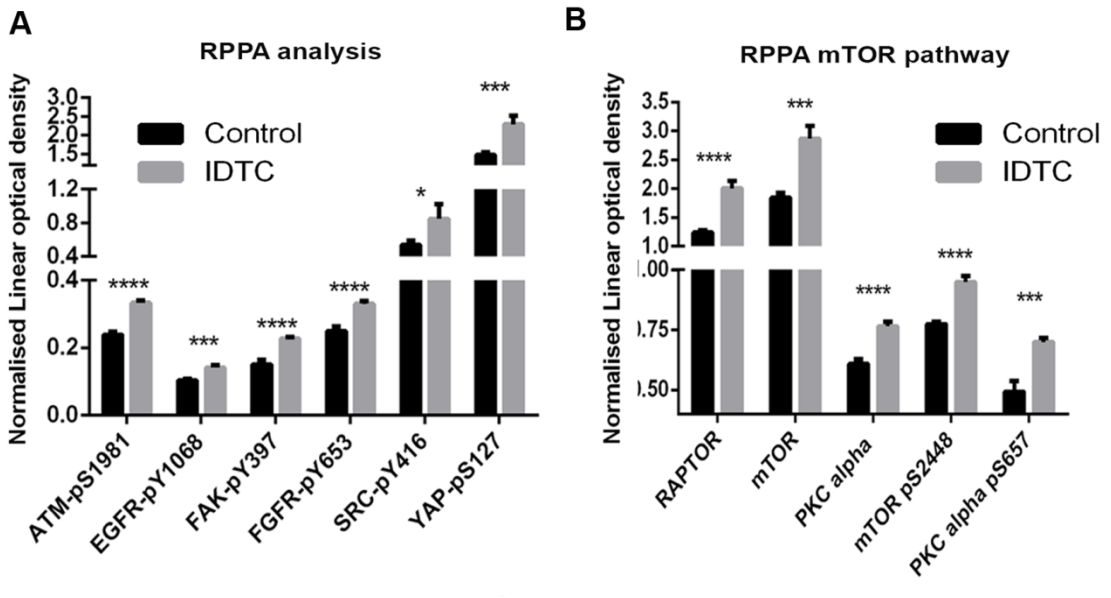


***Figure 55: IDTCs show activation of multiple signalling pathways**. (A,B) RPPA analysis depicting normalised linear optical density of proteins in WM164 parent (Black) and IDTCs (Grey) lysates plotted as bar graphs., Error bars represent the standard deviation from the mean. Statistical analysis was done using the T test and the P value is represented by (*) where P</= 0.0001 (****) , P</= 0.001 (***),, P</= 0.01 (**) and P</= 0.05(*) RPPA data provided by Gao Zang).*

The FAK-SRC axis has been reported to be a key driver of migration and metastasis, in melanoma and other cancers (Hiratsuka et al., 2011; Li et al., 2001) . Other than that IDTCs also showed an increase in RAPTOR and mTOR expression along with an increase in mTOR pS2448 and PKCA pS657 phosphorylation (Figure 55 B), which is a known downstream target of the mTOR pathway. PKCA is further reported to induce AKT and ERK activation (Haughian et al., 2009). They further provide evidence for the capability of IDTCs to exert a considerable level of drug tolerance making them difficult to be targeted.

(2.2.6) Induced drug tolerance is a common phenomenon applicable to multiple cancer types and CD271 a potential marker for identifying early drug tolerant cells

The occurrence of IDTCs as an early response towards drug exposure in melanoma advocates for an overall model of early drug tolerance in other cancer entities. To test this A549 lung cancer cells and MCF7 breast cancer cells were exposed to a

low dose of cisplatin (5µM) for a period of 12 days with 133(+/- 5.65)% A549 and 142(+/- 1)% of MCF7 cells surviving the exposure (Figure 56).

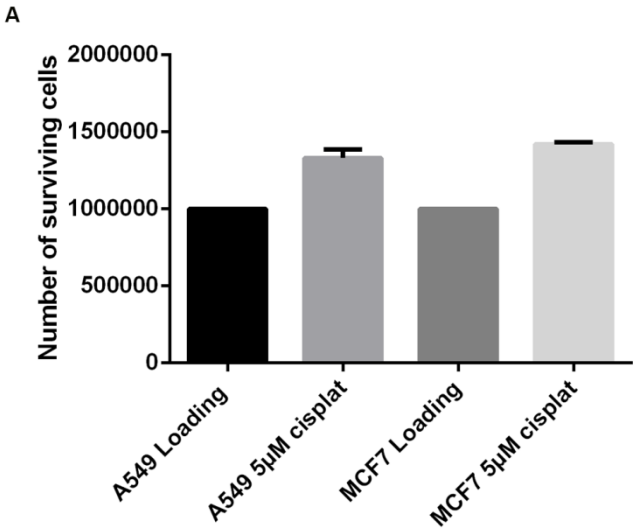


Figure 56: IDTC induction in A549 and MCF7. *1x10^6 A549 and MCF7 cells were plated in duplicate for each time point and drug dosage, and exposed to DMSO and Cisplatin at 5µM for 12 days and the number of viable cells was counted with the casy cell counter from two experimental duplicates and represented as bar graphs. Error bars represent thre standard deviation from the mean.*

Like in melanoma cells the surviving population of A549 (Figure 57 A) and MCF7 (Figure 57 B) cells showed a substantial increase in CD271 as well as CD31 expression. Moreover I also observed an increase in ALDH activity in the population of both, A549 (Figure 58 A) and MCF7 (Figure 58 B). By probing their sensitivity towards different cytotoxic drugs multiple drug tolerance was observed (Figure 7C and D). Similar to melanoma cells, this suggests a transition into an early drug tolerant - IDTC state.

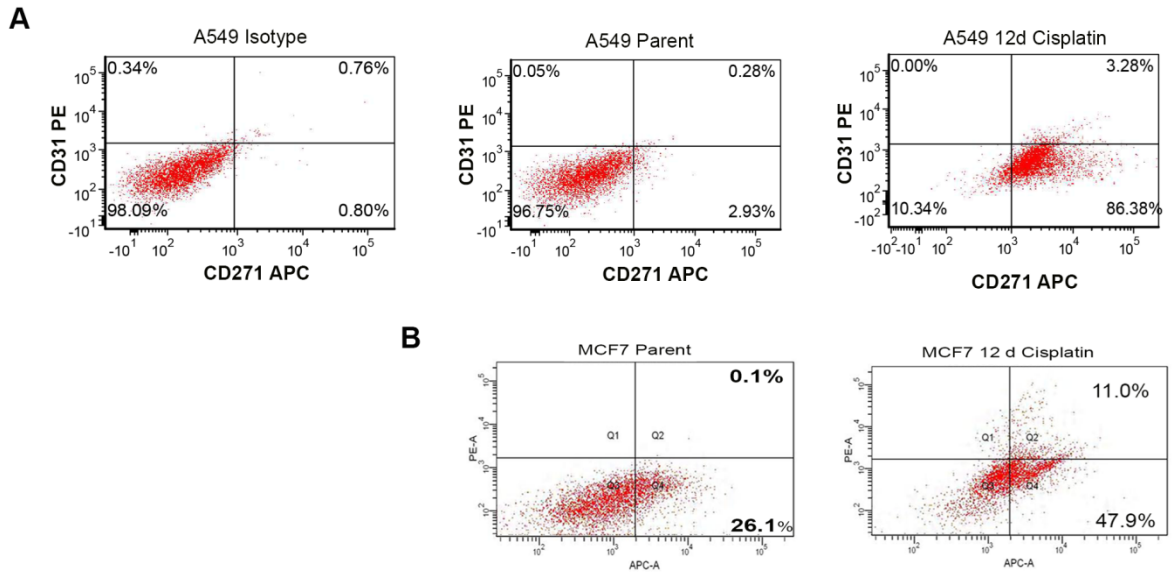


Figure 57: Moderate drug exposures lead to an IDTC signature in other cancer types*. (A,B) CD271 and CD31 expression of A549, MCF7 parent, and IDTCs (exposed to 5µM cisplatin for 12 days) subjected to flow cytometry analysis.*

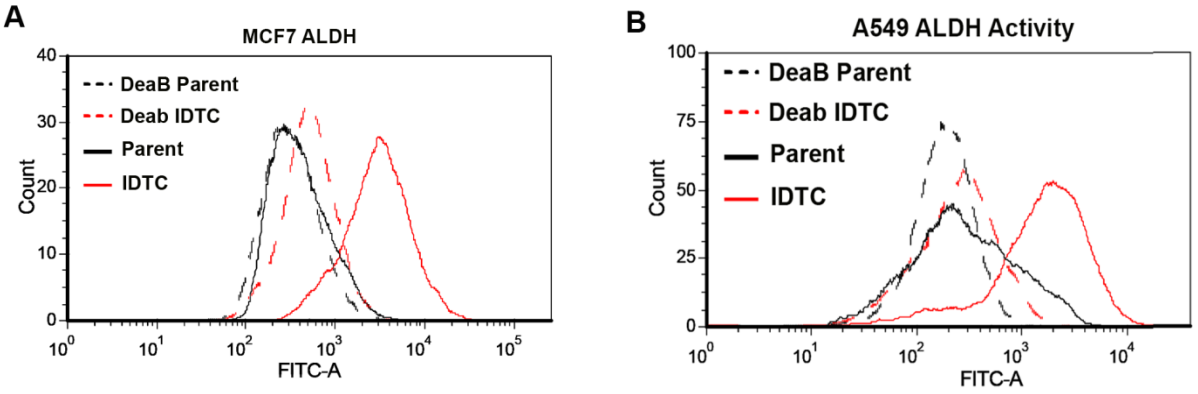
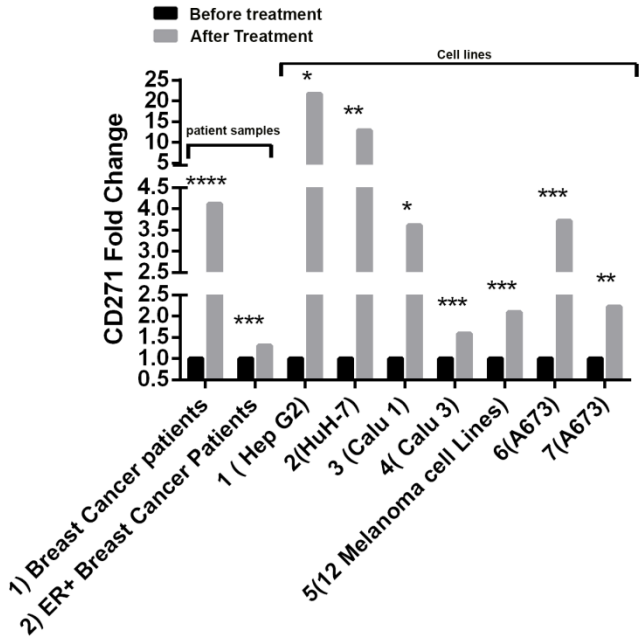


Figure 58: Moderate drug exposure leads to ALDH activity in other cancer types. *(A)ALDH activity of MCF7 parent (Back) and IDTCs (Red) compared to their respective DEAB negative controls. (B) ALDH activity of A549 parent (Black) and IDTCs (Red) compared to their respective DEAB negative controls.*

Predominance of CD271 as a marker for IDTCs prompted us to compare mRNA expression levels of microarray data from independent studies involving patients and in vitro experiments before and after exposure to various drugs based on published evidence. Out of the available data two breast cancer patient cohort studies matching our criteria for early drug response, one involving 32 patients receiving a neo-adjuvant chemotherapy with Epirubicine® at 90mg/m^2 and cyclophosphamide at 600mg/m^2 for 84 days following intermittent dosing and another involving 18 patients receiving 2.5mg/day letrozole for 12-14 days, both of which reported a significant increase in CD271 expression after treatment (Figure 59).



Patient Data

	Study	Total Samples	Treatment	Access number	Time	p-value
1	Paired Invasive breast cancer tissue neoadjuvent chemotherapy after vs before	32	4 cycles of epirubicine 90 mg/m^2 and cyclophosphamide 600 mg/m^2 every 3 weeks	GEO:GSE21974	84 days	0,000005
2	paired ER positive breast cancer after lerozole treatment vs before	18	2.5mg /day letrozole	GEO:GSE10281	12-14 days	0.0006

Cell Line Study

No	Cell Line Study	Cancer type	Access Number	p-Value
1)	HepG2 10μM 5-aza-2-deoxy cytidine 5 days vs untreated	Liver	GEO:GSE35311	0.038
2)	HUH-7 10μM 5-aza-2-deoxy cytidine 5 days vs untreated	Liver	GEO:GSE35311	0.008
3)	calu1 7 days with 24 hr treatment 1μM 5-aza-2-deoxy cytidine(day 1,3,5),10nM TSA(day 5) vs untreated	Squamous cell lung cancer	GEO:GSE14315	0.012
4)	calu3 7 days with 24 hr treatment 1μM 5-aza-2-deoxy cytidine(day 1,3,5),10nM TSA(day 5) vs untreated	Adenocarcinoma lung cancer	GEO:GSE14315	0.001
5)	Melanoma cell lines(average of 12) treated with 5μM 5-aza-2-deoxy cytidine+4hrs 300nM TSA(day 3) vs untreated	Melanoma	GEO:GSE32492	0.001
6)	A673 doxorubicin 59.88 nM 5 days Vs DMSO	Ewing Sarcoma	GEO:GSE6930	0.0004
7)	A673 cytarbine 0.34μM 5 days Vs DMSO	Ewing Sarcoma	GEO:GSE6930	0.005

Figure 59: CD271 increase after therapy could be a generic phenomenon. *Publicly available microarray data sets from Gene Expression Omnibus (GEO) showing an increase in CD271 expression (left) after treatment in various conditions as mentioned in the table (right) analysed by using next bio meta-analysis. P value is represented by (*) where P</= 0.0001 is represented by (****) , P</= 0.001(***),, P</= 0.01(**) and P</= 0.05(*).*

Several in vitro studies including liver, lung, Ewing sarcoma and 12 different melanoma cell lines with different drug types also showed similar results, suggesting that an increase in CD271 expression might serve as a common marker for predicting an IDTC state.

(2.3)Discussion

The studies I performed shows that cancer cells have an innate ability to respond to persistent hostile conditions by exhibiting an early primary response corresponding to a primary state of drug tolerance (Figure 8A).It seems that the cancer cells further gradually progress to permanent resistance from this primary drug tolerant state due to mutations or epigenetic modification as described by multiple studies which explain permanent drug resistance(Emery et al., 2009; Engelman et al., 2007; Villanueva et al., 2010). This response seems to be generic in nature following

exposure to different adverse conditions like nutrient starvation, drug exposure or hypoxia. Some of the observations are similar to what has been reported previously on the presence of cancer stem cells but the inferences are different.

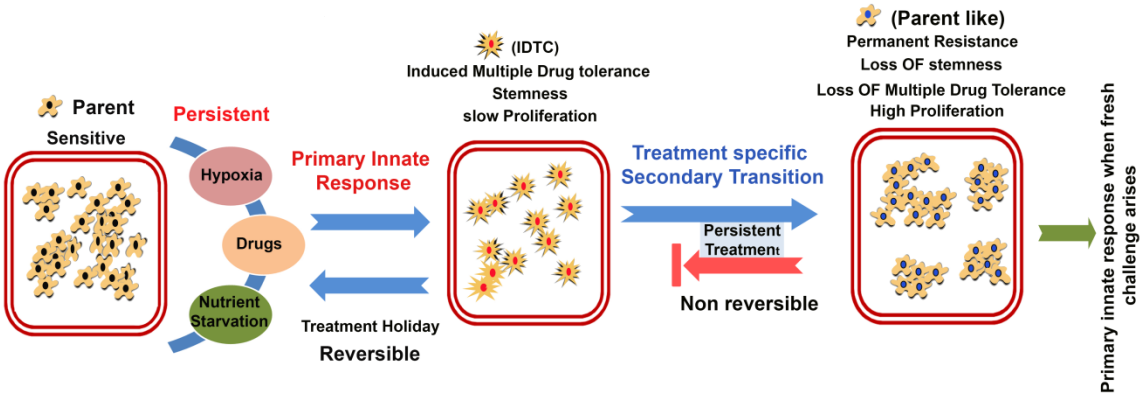


Figure 60.Proposed General model for acquired drug resistance whereupon different conditions induce the formation of IDTCs, and surviving IDTCs under consistent drug exposure lead to the transition into a permanently drug resistant state.

The cancer stem cell model predicts the selection of a clonally distinct subpopulation of cells, in order to explain the survival of a particular drug resistant population. This prediction is based on the assumption that a predefined subpopulation of parent cells with stem cell characteristics lacks sensitivity towards the drug they are exposed to, leading to their survival and relapse of the disease. The data, however, indicates that exposure to the drug itself after a relatively short period of time induces a stem cell like phenotype in the parent population. One obvious argument for the selection of a particular subpopulation regarding the cancer stem cell model could be the observation of massive cell death in the parent population at high drug concentrations. Whereas even at lower drug concentrations I could observe the same cancer stem cell-like phenotype being induced in the surviving cells.

For melanoma the clonal selection and enrichment of an ABCB5 and CD271 highly tumourigenic subpopulation might meet these criteria, which has been previously described after drug exposure in vitro (Frank et al., 2005; Frank et al., 2010) and in patients (Chartrain et al., 2012; Shiota et al., 2013). The basic concept has also been expanded and slightly modified by another proposed drug resistance model, which seems to be applicable for multiple cancer types, described as the chromatin-mediated drug resistance model (Sharma et al., 2010). The essential deviation from the cancer stem cell model encompasses a pre-existing drug resistant subpopulation, transient in nature. Even though these two models are decisive in

terms of the drug resistance of the particular subpopulation, they are inconclusive on the basic concept behind the mode of their evolution.

The IDTC state represents a convergence of both the stem-like and the chromatin mediated drug resistant conditions. This includes an increase in ALDH activity (Croker and Allan, 2012) and various cancer stem cell markers like CD271, ABCB5, CD44 and a high tumourigenic potential, a hallmark of cancer stemness. The signatures of chromatin mediated drug resistant cells have been reported to be the loss of H3K4me3 and an increase in H3K4 demethylases like KDM5A or KDM5B, which was found in IDTCs as well along with a loss in H3k27me3 and an increase of H3K9me3, suggestive of a global chromatin remodelling not limited to demethylation at H3K4.

The high tumourigenic potential of IDTCs critically ensures that a low number of cells that survive a harsh environmental condition could constitute the seed to form tumours once the conditions are favorable for their growth and propagation. Expression of CD271, which has largely been linked to define a melanoma initiating population (Boiko et al., 2011) was found to be a general marker of the IDTC state in several cancer types, indicating a possibility of expansion of its use as a biomarker.

A variety of efforts have been undertaken to identify methods to target slow growing subpopulations, which has been identified by distinct markers like KDM5B, KDM5A or CD271.Iobserved that the combination strategies including the use of HDAC, IGF1R, Pi3K/AKT inhibitors (Sharma et al., 2010) and oligomycin A targeting mitochondrial survival pathways (Roesch et al., 2013), along with the primary drug exert a higher potential to eliminate the parent population before transition into IDTCs, whereas the combinations did not show a substantial effect in the cells already in the IDTC state.

Even the knock down of melanoma stem cell genes like CD271 or KDM5B, which are reported to exterminate the slow cycling multiple drug tolerant melanoma cell population, could only increase the sensitivity of parent population to drug exposure. While mild dosage of drugs could still instigate the transition in these cells leading to IDTC population which still remained multiple drug tolerant. Cells in the IDTC state express drug efflux molecules like ABCB5. Accordingly, I observed IDTCs being more competitive in effluxing chemotherapeutic drugs, like doxorubicin if compared to the parent population (data not shown), while I could not observe them to have a significant role in mediating drug resistance to small molecular inhibitors like

GSK1120212. This observation is in line with previous report which has also described lack of drug efflux against EGFR inhibitors in chromatin mediated drug resistant cells (Sharma et al., 2010).

The drug tolerance characteristics of IDTCs seem to be a result of activation of multiple signaling cascades, many of them being simultaneously capable of activating ERK and AKT survival pathways. This includes the activation of ATM (pS1981) (Khalil et al., 2011), EGFR (pY1068) (Grant et al., 2002; Saito et al., 2004), FGFR (pY653) (Mohammadi et al., 1996; Zou et al., 2012),YAP (pS127) (Overholtzer et al., 2006), FAK (pY397) (Glover et al., 2004) and SRC (pY416) (Kim et al., 2009a) along with mTOR signaling (Haughian et al., 2009). IDTCs are therefore capable to regain their AKT and ERK signaling by compensating through other pathways, thereby splitting the burden of activation of the ERK-AKT survival pathways between various molecules. This also explains why targeting of individual molecules like CD271 or KDM5B could not eradicate the transition but rather only reduce the number of IDTCs generated due to drug exposure. Accordingly the role of many of these signaling cascades in cancer drug resistance has been well known, including mTOR signaling (Jiang and Liu, 2008), FGFR signaling (Dieci et al., 2013), ATM signaling(Xu et al., 2013), YAP signaling (Huo et al., 2013) and SRC signaling (Girotti et al., 2013). Even though targeting each of these pathways seems to be rational, simultaneous targeting may not constitute a clinically viable option. Therefore I propose that interference with the primary transition from parent cells to IDTCs might be a better strategy.

Transposing this concept to the in vivo situation would mean that IDTC like cells are almost certainly already present due to adverse conditions imposed by the tumour micro-environment and improper drug kinetics. Continuous administration of drugs thus inevitably will lead to permanent drug resistance. Hence employing an intermittent dosing is a timely concept (Figure 61 A), which already has been proven to delay the emergence of resistance in patients treated with BRAF inhibitors (Das and Stuart, 2013). Combining the intermittent drug dosage with previously described combination therapies or targeting CD271 and KDM5B like molecules could lead to a better result, as this strategy gradually leads to the depreciation in tumour volume alongside with a fairly reduced number of cells transforming into IDTCs in the course of the treatment (Figure 61 B).

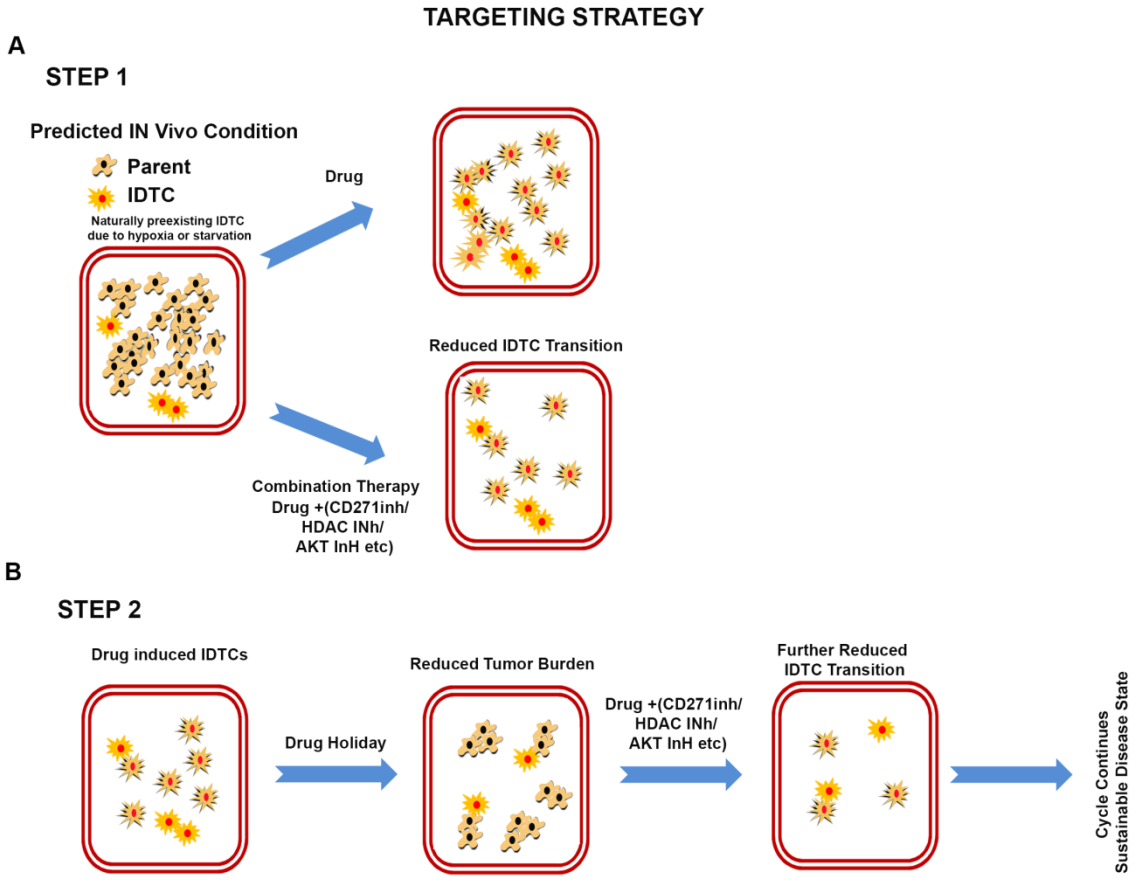


Figure 61: Targeting IDTC for a sustainable disease state

(A) Strategic approach of combinating drugs to reduce the number of cells that allow transition into the IDTC state as opposed to conventional strategy.

(B) Repeating the strategic approach with a drug holidays to ensure consistent shifting of the IDTC state and reduction of tumor volume

Further testing of this concept is necessary to strategize treatment regimens. Intensive screening of multiple cancer types in IDTC state is required for better understand common targets that could be used in combination therapies with cancer specific drugs.

An evolutionary perspective of our studies points to a deviation from the classical Darwinian model of evolution, which had been widely used to predict the occurrence of cancer drug resistance (Gerlinger and Swanton, 2010; Gillies et al., 2012). The model is closely reminiscent to the theory proposed long ago by James Mark Baldwin, known as the Baldwin effect, underscoring the significant role of phenotypic

plasticity (Baldwin, 1896). Our observations, however, do not follow the genetic assimilation theory or the Waddington effect as the innate response did not lead to a population which permanently exhibited plasticity in the absence of drugs, rather plasticity seems to be exhibited as a trait when needed (Crispo, 2007). It is arguable that certain features presented during the IDTC state can be carried over to the resistant state contributing to the emergence of resistance. That being said, after the emergence of the IDTC state a Darwinian process may overtake the course of evolution driving a permanently resistant phenotype from the IDTC population, where mutations or epigenetic changes could provide selective growth advantages.
The model I am proposing combines several features of already existing concepts of drug resistance but provides an inimitable scaffold of a stage dependent process culminating in permanent resistance. Thus it might serve as a valuable tool for adjusting therapeutic strategies in order to subvert a relapse in patients.

(2.4) Materials and Methods

(2.4.1)Generation of IDTCs

IDTCs were generated by exposing cancer cells to PLX4032, Cisplatin, Low glucose or hypoxic conditions for a minimum of 12 days. RPMI media supplemented with 2mM L –Glutamine, 2x Penstrep and 5 % FCS were used to grow the cells. Low glucose media was made by mixing RPMI normal media in 1:4 ratio with RPMI no Glucose media. Hypoxic experiments were done in Hypoxic work station facility (Biospherix (Model nr.: G300CL, BioSpherix, Lacona, NY, USA)) with the *Ludwig Boltzmann* Institute for Lung Vascular Research, ZMF. The media was replenished every 3rd day for the period of experiments.

(2.4.2)Gene expression analysis

Gene expression analysis were carried out as previously reported (Cvitic et al., 2013) by the Division of Core Facility for Molecular Biology at the Centre of Medical Research at the Medical University of Graz, Graz, Austria. Briefly Ambion WT Expression Kit for Affymetrix GeneChip Whole transcript (WT) Expression Arrays (Life Technologies; Carlsbad, CA, USA) was used to label total RNA. GeneChip Human 1.0 ST arrays was used for hybridization according to manufacturer's

instruction.250 ng of total RNA was used for the experiments. Affymetrix GeneChip scanner GCS3000 was used for reading. Genomic Suite v6.5 (Partek Inc, St Louis, MO, USA) was used for analysis of the results. All samples passed the quality control check. Further The data generated were loaded to in to Ariadne pathway studio (Elsevier) for pathway analysis.

(2.4.3) RPPA analysis

These experiments were done in collaboration with DR. Meenhard Herlyn, Wistar Institute, Philadelphia. Cell lysates were prepared in Lysis Buffer(1% Triton X-100, 50mM HEPES, pH 7.4, 150mM NaCl, 1.5mM MgCl2, 1mM EGTA, 100mM NaF, 10mM Na pyrophosphate, 1mM Na3VO4, 10% glycerol, containing freshly added protease and phosphatase inhibitors from Roche Applied Science (Cat. # 05056489001 and 04906837001). The lysates were mixed with SDS buffer without bromophenol blue after calorimetric protein determination and boiled for 5 minutes and were kept frozen at -80 until analysis.

Cell lysates were two-fold-serial diluted for 5 dilutions (from undiluted to 1:16 dilution) and arrayed on nitrocellulose-coated slide in 11x11 formats. Samples were probed with antibodies by CSA amplification approach and visualized by DAB colorimetric reaction. Slides were scanned on a flatbed scanner to produce 16-bit tiff image. Spots from tiff images were identified and the density was quantified by Array-Pro Analyzer. Relative protein levels for each sample were determined by interpolation of each dilution curves from the standard curve of the slide (antibody). All the data points were normalized for protein loading and transformed to linear value which is represented as bar graphs.

(2.4.4) Angiogenesis array

Expression profile of angiogenesis related cytokines and growth factor were analyzed with Proteome Profiler Human Angiogenesis Array Kit (ARY007-R&D systems) according to the manufacturer's protocol. Briefly Parent and IDTC cells in 6 well plates were incubated with 1ml FBS free RPMI media for 16 hours and further isolated and spin down to remove any cell debris. The total protein content of the samples was analyzed using Bradford and protein equalization was carried out before loading into the antibody strips provided. In order to be able to compare the

results the assays were carried out simultaneously and were developed simultaneously in the same x ray film in order to ensure the same exposure time. Further the x ray films were scanned using Canon 8400F scanner and pixel density was analyzed using image J software.

(2.4.5) In Vivo tumourigenesis assay

NOD.CB17-Prkdcscid/J were separated into groups receiving injections of 50, 500 or 5000 of WM 164 Parent (Neck),PLX4032 500nM IDTC (Left Flank) and Resistant cells(Right Flank) in a suspension of Matrigel (BD Matrigel™ Basement Membrane Matrix, Growth Factor Reduced) with complete media without FCS in 1:1 ratio. Then the tumor development was monitored in each group every week once for the period of experiments and tumor volume was measured using caliper by applying the formulae(width x length x height)/2. Mice were sacrificed after the experiment period or when the cumulative tumor volume had reached the ethical limit. Tumor tissues were formalin fixed and paraffin embedded and the slides were further stained for CD31 expression. The work was carried out under the ethical guidelines of Bundesministerium fur wissenschaft und forschung(B.M.W_F:GZ66.010/0003-11/3b/2011)

(2.4.6)Sphere formation assay

50, 500 or 5000 WM164 melanoma cells were suspended in 500µl serum free Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham media(Invitrogen) supplemented with 10 ng /ml FGF2 (Cell signaling #8910) and 10ng/ml EGF(Cell signaling #8916), which has been a modification to previously reported protocol to produce more stringent conditions where sphere formation of IDTCs and parent cells could be compared in the absence substantial external stimuli from B 27 like stem cell supplements(Mo et al., 2013), which is shown to aid melanoma sphere formation. Further the cells were plated in ultra-low attachment 24 well cell culture plate and media was replenished every 3rd day with additional 500 µl and sphere formation was assessed after 1 week period.

(2.4.7) Endothelial cell invasion assay

Endothelial cell invasion properties of IDTCs were compared with parent population using BD BioCoat™ Angiogenesis System for Endothelial Cell Invasion (Cat:08-774-

378). Briefly the cells were isolated and $2x10^4$ IDTCs or parent cells were inoculated in serum free media and plated in the upper chamber ,while the lower chamber were inoculated with 10% FBS RPMI media and the invasion of the cells were monitored for the next 72 hrs. Then further cells in the bottom chamber were labeled by Vybrant CM-DiI cell-labeling solution , Invitrogen, Cat. No.: V22888 and visualized under microscope. The experiments were repeated in triplicates and the number of cells from 3 different 10 x field per experiment where counted for analysis.

(2.4.8) Histone isolation

Epiquick total histone extraction kit (OP-0006, Epigentek), was used according to manufacturer's protocol. Briefly The IDTCs and parent cell pellets were re-suspended in pre-lysis buffer and after incubation supernatant was removed and pellet was re-suspended in lysis buffer, followed by extraction of lysates. The lysates pH were further compensated by balancing buffer and total protein content was determined and further subjected to western blot analysis.

(2.4.9) Antibodies

Antibodies directed against KDM5B, pIGF1R, pAKT, pan-AKT, p44/42 ERK1/2, total ERK ½ and p217/221MEk1/2 were from Cell Signaling Technology. The CD271 antibody used for immunohistochemistry of paraffin-fixed slides was purchased from Abcam. FACs antibodies CD271 APC was from Mitenyl Biotech and CD31-PE was from BD Bioscience. The H3K4me3, H3k27me3, H3k9me3 and H3 antibodies were purchased from active motif.

(2.4.10) Inhibitors

PLX4032, GSK1120212, Linsitinib (OSI-906),Cispaltin, Adrucil,Docetaxel and TSA were purchased from Selleckchem. Ly294002 was purchased from Cell Signaling Technology.

(2.4.11) Cell lines

BRAF mutant melanoma WM164, A375 and 451 Lu cells were previously obtained Wistar Melanoma cell lines. A549 and MCF7 cell lines were obtained from ZMF cell line facilities.

(2.4.12) Long term cell survival crystal violet staining

Cells after drug treatment at various time points were fixed with 4% paraformaldehyde, followed by 30 min incubation with 0.5% crystal violet solution. The plates were then washed and pictures were taken using a microscope and gel documentation unit after drying.

(2.4.13) Flow cytometry analysis of cell surface markers

Expression of cell surface markers was checked after incubating the respective antibodies with Macs Miltenyl staining solution (Rinsing solution with 0.5% BSA stock) for 20 min followed by a wash. In case of double staining the procedure was repeated with the second antibody. CD31 and CD271 antibodies were in concentrations according to user manual. The samples were analysed with BD LSR II Flow Cytometer from the ZMF FACS core facility.CD31-PE was measured using 488/Red fluorescent channel. CD271 APC was measured using 633/APC fluorescent channel. Individual compensations were done prior to analyse.KDM5B promoter activity was analysed in 488/Red fluorescent channel.

(2.4.14) ALDH activity analysis.

ALDH activity was analysed using ALDEFLUOR™ Stem Cell Identification & Isolation kit from stem cell technologies according to the user manual. Briefly cells were split in to 2 and incubated with ALDEFLUORTM assay buffer having efflux inhibitors. One set was pre incubated with 20 microliter of DEAB for 2 mins as negative control and both sets were subjected to incubation with ALDH-substrate, BAAA ((BODIPY®-aminoacetaldehyde) for 20 mins. Further the cells were washed with ALDEFLUORTM assay buffer and analysed with BD LSR II Flow Cytometer from the ZMF FACS core facility in 488/Green fluorescent channel.

(2.4.15) Caspase 3 apoptosis assay

The Caspase 3 apoptosis assay was carried out using a PE Active Caspase-3 Apoptosis Kit from BD Pharmigen, according to manufacturer's protocol. Briefly, cells after treatment were washed and permeabilized with cytofix/cytoperm solution followed by washing and reincubation with Caspase 3 antibody and were further

analyzed by flow cytometry. All experiments were done in triplicate and graphs represent mean values with error bars representing standard deviations.

(2.4.16) MTT assay

$2x10^4$ cells were seeded in 96-well culture plates for the times mentioned in the experiments. They were subsequently incubated with MTT (3-(4, 5-dimethylthiazolyl-2)-2,5 diphenyltetrazolium bromide) reagent (10 µl) at 37ºC for 3 h. 100 µl detergent reagent was then added to the wells followed by incubation in the dark at room temperature for 3 hr. Absorbance was recorded at 570 nm using a microtiter plate reader. All experiments were done in triplicate or duplicate and graphs represent mean values with error bars representing standard deviations.

(2.4.17) Lentiviral vectors

Sh CD271 (p75NTR) and Sh KDM5B (PLU1) Sh RNA pools were ordered from Santa Cruz Biotechnology and transduction were carried out according to the manufacturer's protocol briefly cells were incubated with 8ng/ml polybrene complete media and incubated overnight with the lentiviral particles and selected wit 10µg/ml puromycin for 1 week period.

(2.4.18) RT PCR

Total RNAs were isolated using a QIAGEN kit according to the manufacturer's protocol and 1 µg of RNA was used to generate cDNA using a RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Life Sciences). The SYBR Green Master Mix from Applied Biosystems was used for PCR amplification and products were detected by AB7900 Standard real time PCR system (Applied Biosystems). *18s* RNA was used as an internal control and relative gene expression levels were calculated as delta CT.

RT PCR Primers

	Forward	Reverse
OCT4	CTTCTGCTTCAGGAGCTTGG	GAAGGAGAAGCTGGAGCAA
KDM5A	CCAGCCTGATCTTCTGCATC	CCAGCACACTGATTGGTCCT

KDM5B	TCATTTTACGCCACGTATCCA	CTTTGCAATCTGGTCCAAGAA
SOX10	ACACCTTGGGACACGGTTTT	GGTCCTCGCAAAGAGTCCA
ABCB5	AGGGAAGCAAATGCGTATGA	GCTCCTTTTTCCCCTACCAA
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

(2.4.19) In vivo low dose CD271 induction.

These experiments were done by our collaborators in Wistar institute, Philadelphia, USA. NOD SCID IL2 receptor gamma chain knockout (NSG) mice were inoculated subcutaneously with $1x10^6$ 451Lu human melanoma cells in a suspension of Matrigel (BD Matrigel™ Basement Membrane Matrix, Growth Factor Reduced) / complete media at a ratio of 1:1. After tumours had reached a volume of 200-300 mm^3, mice were randomized into groups of five. The groups were then treated with either vehicle (5% DMSO, 1% methylcellulose in dH_2O) alone or with PLX4720 at a dose of 15 mg/kg or 30 mg/kg BID per oral gavage. The tumours were measured twice weekly using calipers and volumes were calculated as (width x length x height)/2. Mice were sacrificed after 2 weeks of treatment and tumours were harvested 4 hours after the last dose. Tumour tissues were formalin fixed and paraffin embedded and the slides were further stained for CD271 expression. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Wistar Institute Animal Care and Use Committee (Protocol Number: 111954).

(2.4.20*) Immunohistochemistry* (Done by collaborators department of pathology)

Samples of formalin-fixed paraffin embedded tissues were retrieved from the archives of the Department of Dermatology, Medical University of Graz according to ethical guidelines. Briefly, slides were deparaffinized and antigen retrieval was done using 0.01 M sodium-citrate buffer (pH 6.0) and the specimens were then blocked with 1% H_20_2 in methanol. The slides were further incubated with the CD271(Abcam mouse monoclonal ab3125) or CD31 (Thermo scientific, USA)followed by incubation with biotinylated anti-mouse antibody (Dako 5001, ready to use) Slides were further

incubated with the streptavidin peroxidase detection system (Dako 5001, ready to use) and developed with Dako Real DAB+ chromgen (Dako 5001, 1:50).

(2.4.21) Immunocytochemistry (Done by collaborators department of pathology)

These experiments were done by our collaborators in Department of pathology, Medical university of Graz. WM164 control and ADTC cells were grown in poly D lysine BD biocoat chamber slides (BD Bioscience). A monoclonal antibody to Mart-1/Melan A Cocktail (Biocare Medical CM077B) was used as the primary antibody (1:50 with antibody diluent Dako S202230). Slides were pretreated for 40 min in a water bath (98.5°C) with an epitope retrieval kit (Dako K500111), and then left for 20 min at room temperature. Blocking was performed with a peroxidase blocking solution (Dako S202386). Staining was detected by a Dako Real Detection Kit (Dako K500111) and AEC (Dako 346430) as a chromogen. Tyrosinase staining was carried out similarly with a Tyrosinase antibody (Novocastra, NCL-L-TYROS) and epitope retrieval was done with Target Retrieval Solution, Dako S236784 with a DakoEnVision Detection Kit (Dako K500711).

(2.4.22) Public micro array data set analysis

Next Bio Meta-analysis search (www.nextbio.com) was used to identify data sets comparing the expression of CD271 (p75NTR) levels after treatment to before treatment from multiple independent cancer studies. The p Value Cut off for studies to include in the analysis was set to 0.05 with the minimum-fold change to be ± 1.3. Further details of the data sets were incorporated from GEO or EMBL websites. Each bar in the graph shows the CD271 expression in the treated group compared to the control.

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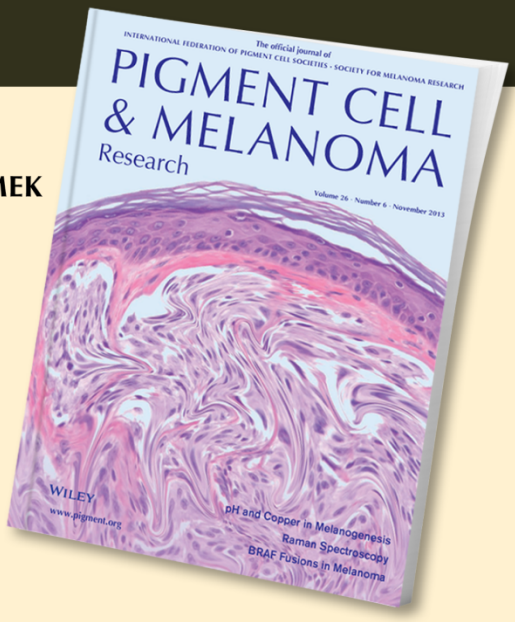

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Pigment Cell Melanoma Res. **26**; 912–916 SHORT COMMUNICATION

TGF-β1 and TNF-α differentially regulate Twist1 mediated resistance towards BRAF/MEK inhibition in melanoma

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Summary

Resistance to BRAF and MEK inhibition is a common phenomenon in melanoma. Cytokines and transcription factors have been attributed to contribute to the loss of sensitivity towards these inhibitors. Here, we show that transforming growth factor (TGF)-β1 if combined with PLX4032, a BRAF inhibitor, or GSK1120212, a MEK inhibitor, substantially increased cell death in BRAF-mutant melanoma cell lines. This increase was based on the combined regulatory decrease in Twist1, an antiapoptotic protein. Overexpression or silencing of Twist1 attenuated or aggravated induction of apoptosis through PLX4032 or GSK1120212, respectively. Exposure to tumour necrosis factor (TNF)-α, however, led to increased Twist1 levels and oppositional decrease in cell death if exposed to PLX4032 or GSK1120212. This increase in drug resistance again depended on Twist1 levels. Our studies suggest that Twist1 as a common downstream target of multiple signalling cascades plays a crucial role in mediating drug resistance to BRAF- and MEK-targeted molecular inhibitors.

Targeted molecular cancer therapy with the BRAF inhibitor Zelboraf® (Vemurafenib; PLX4032) showed a dramatic improvement in BRAF-mutant melanoma patients with advanced disease (Flaherty et al., 2010). The results were similar with administration of molecular inhibitors targeting MEK1,2 (Flaherty et al., 2012), a downstream target of BRAF. However, drug resistance has been an equally prevalent phenomenon in these patients (Johannessen et al., 2010; Nazarian et al., 2010; Villanueva et al., 2010). It is very well known that cytokines and growth factors from the tumour environment contribute to melanoma progression (Lazar-Molnar et al., 2000). Recently, hepatocyte growth factor (HGF) has been shown to be involved in resistance to the BRAF inhibitor Zelboraf®, a finding, which underscores the importance of cytokines and growth factors found in the tumour environment and secreted by cancer cells alike contributing to drug resistance (Straussman et al., 2012; Wilson et al., 2012). Corresponding to these observations, there have been multiple reports in recent years that have emphasized the crucial role of Twist1, an epithelial–mesenchymal transition regulator, in the development of

Significance

Drug resistance to small molecular targeting inhibitors, like BRAF and MEK, is considered to depend on both intrinsic and extrinsic factors. We here show the importance of Twist1 as an antiapoptotic protein and as a common downstream target of ERK and STAT3 signalling, playing a crucial role in determining drug resistance to BRAF and MEK inhibitors. Dynamic regulation of Twist1 by extrinsic factors, like TGF-β1 and TNF-α, sustainably affects the response of BRAF-mutant melanoma cells to these inhibitors.

drug resistance (Kwok et al., 2005; Li et al., 2009) and melanoma progression (Koefinger et al., 2011; Weiss et al., 2012).

To identify potential cytokines contributing to the loss of sensitivity towards BRAF or MEK inhibitors, the effect of several growth factors in this context was tested (data not shown). We observed that TGF-β1, a context-dependent tumour-suppressive or growth-promoting cytokine, significantly affected the response of melanoma cells to both BRAF and MEK inhibitors. Treatment of WM164 cells with PLX4032 resulted in caspase-3-mediated apoptosis, while the same inhibitor in combination with 5 ng/ml of TGF-β1 almost doubled the induction of apoptosis (Figure 1A). The effects were also similar with GSK1120212, a MEK inhibitor, (Figure 1A) which corresponded to the relative cell viability (Figure S1A). The results were consonant in other BRAF V600E–mutant melanoma cell lines SKMEL28 (Figure 1B) and WM9 (Figure S1B). Correspondingly, inhibition of the TGF-β1 receptor with SB431542 partially rescued cell death induced by PLX4032 and its combination with TGF-β1, suggesting that endogenous TGF-β1 produced by melanoma cells also contribute to the induction of

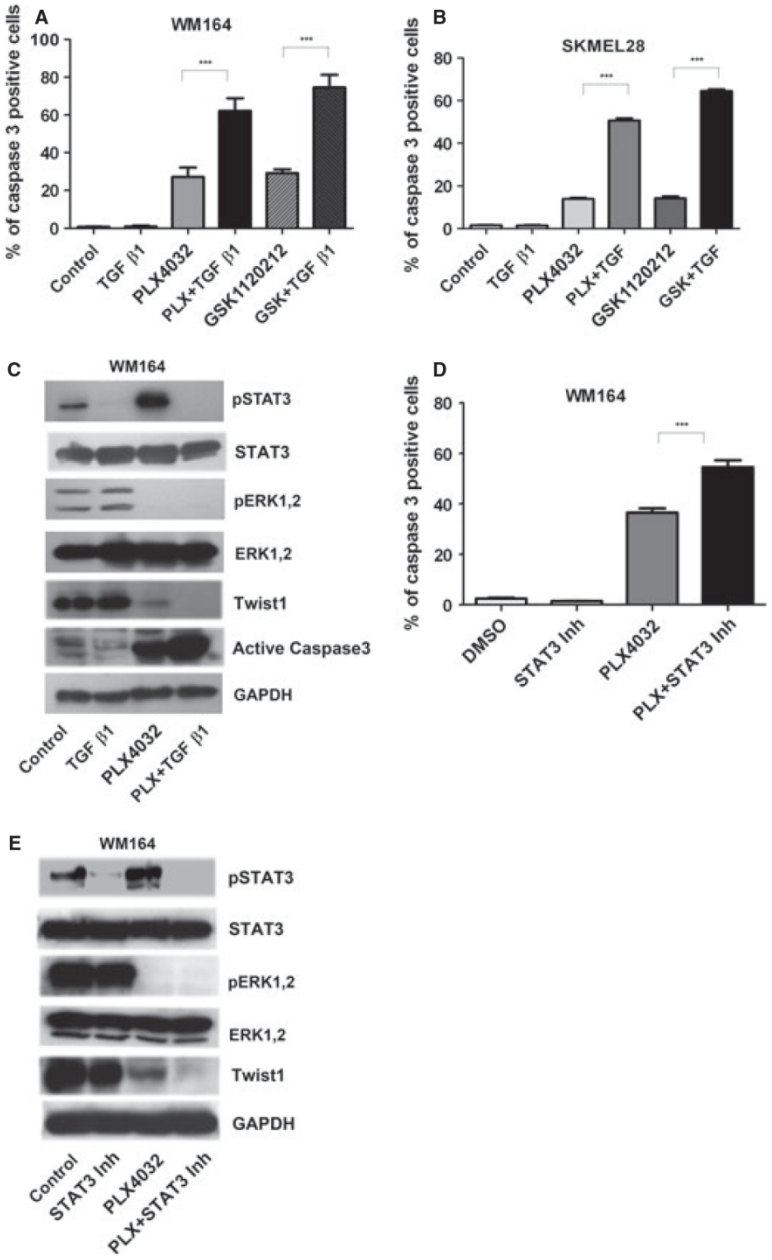

Figure 1. Percentage of cleaved caspase-3-positive cells after exposure to a BRAF (PLX4032 1 μM) or MEK inhibitor (GSK1120212 50 nM) in combination with TGF-β1 (5 ng/ml) for 36 h in WM164 (A) and SKMEL28 (B) (***P-value <0.001- Student's *t*-test). (C) Western blot analysis of total STAT3 and ERK, as well as phosphorylated STAT3 Tyr 705 (pSTAT3) along with ERK phosphorylation (pERK1,2) and its correlation with Twist1 down-regulation and caspase-3 activation in WM164 exposed to the BRAF inhibitor PLX4032 or TGF -β1 alone, or in combination of both, GAPDH is used as a loading control. (D) Percentage of cleaved caspase-3-positive cells after 36 h exposure to BRAF or a STAT3 inhibitor (STAT3 inh VII (1 μM)) alone or in combination (***P-value <0.001- Student's *t*-test). (E) Western blot analysis of total STAT3 and ERK, as well as phosphorylated STAT3 Tyr 705 (pSTAT3) along with ERK phosphorylation (pERK1,2) and its correlation with Twist1 down-regulation in WM164 cells exposed to PLX4032(1 μM) or STAT3 Inh VII (1 μM) alone, or in combination of both, GAPDH is used as a loading control.

apoptosis (Figure S1C). These observations prompted us to study the effect of TGF-β1 in more detail. While analysing the regulation of various epithelial to mesenchymal transition regulators (EMTRs) through PLX4032 and TGF-β1, it was recognized that the combination significantly affected expression of Twist1, whereas expression levels of other EMTRs like Slug and Snail did not change (Figure S1D). Twist1 levels were further lowered by exposure to TGF-β1 combined with BRAF inhibitors (Figure 1C). Mechanistically, TGF-β1 was observed to repress STAT3 Tyr 705 phosphorylation, a known transcriptional activator of Twist1 (Cheng et al., 2008) in line with a previous report (Starsichova et al., 2010). Activation of STAT3 Tyr 705 phosphorylation is known to be leading to the nuclear translocation of the protein and is reported to represent a feedback mechanism in response to ERK inhibition (Decker and Kovarik, 2000; Venkatasubbarao et al., 2005), suggesting that a combination of PLX4032 with TGF-β1 actively blocks the feedback loop, thereby sensitizing the cells for increased induction of apoptosis. Accordingly, exposure to PLX4032 along with a STAT3 inhibitor significantly increased the apoptotic effect (Figure 1D) and also considerably reduced the remnant Twist1 levels (Figure 1E), whereas the STAT3 inhibitor alone did not induce any apoptosis (Figure 1E).

Because both ERK and STAT3 signalling are targeting Twist1 expression, we questioned whether the re-establishment of Twist1 might rescue induced apoptosis by BRAF inhibition. Hence we generated WM164 and WM35 cell lines stably overexpressing Twist1 (WM164-T, WM35-T). WM164-T when exposed to BRAF/MEK inhibitors showed significant resistance to these inhibitors even in combination with TGF-β1 (Figure 2A). A similar trend was also observed in case of WM35-T cells exposed to BRAF inhibition, suggesting that overexpression of Twist1 by itself is capable of partially rescuing cell death induced by BRAF or MEK inhibitors even in combination with TGF-β1 (Figure S2A). Accordingly, WM164 Twist1 stable knockdown cells (WM164 sh-T) showed significantly increased susceptibility to BRAF or MEK inhibition and its combination with TGF-β1 (Figure 2B). Long-term survival of WM164 cells exposed to PLX4032 was also severely affected by the knockdown of Twist1, while overexpression of Twist1 significantly increased the number of surviving cells (Figure 2C) which also corresponded to the residual Twist1 levels (Figure S2B).

From these experiments, we suspected that cytokines that would increase Twist1 expression independent of BRAF or MEK inhibition might lead to the establishment of a drug resistance phenotype against the inhibitors. Hence TNF-α, an inflammatory cytokine which is known to be abundant in the tumour microenvironment (Charles et al., 2009; Popivanova et al., 2008), and has been recently reported to be a potent inducer of Twist1 expression in breast cancer cells, was selected as a potential candidate (Li et al., 2012). On testing, we observed that TNF-α elevates Twist1 levels in BRAF-mutant melanoma cell lines even in the presence of PLX4032 or when combined with TGF-β1 (Figure 3A). Accordingly, addition of TNF-α was sufficient to induce a significant level of resistance to the same inhibitors (Figures 3B and S3A, B). TNF-α also significantly rescued apoptosis induced by a combination of TGF-β1 (Figures 3B and S3A) or STAT3 Inh VII (Figure S3C), with

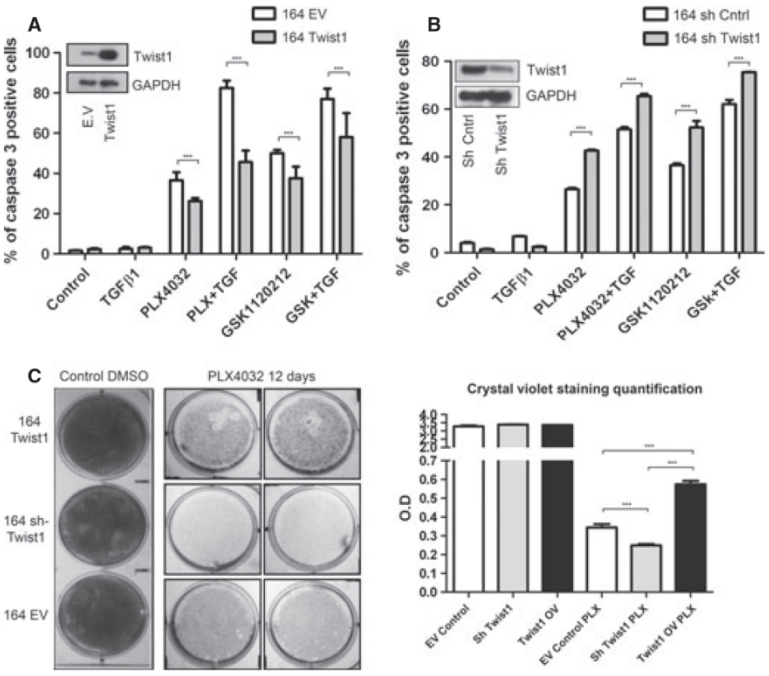

Figure 2. (A) Twist1 overexpression (164 Twist1) in WM164 cells reduces the induction of apoptosis to treatment with PLX4032 (1 μM) and GSK1120212 (50 nM) after 36 h even in combination with TGF-β1 (5 ng/ml) if compared with empty vector control (164 EV) (***P-value <0.001- two-way ANOVA). (B) Twist1 knockdown (164 sh Twist1) sensitizes WM164 to PLX4032 (1 μM) and GSK1120212 (50 nM) induced apoptosis and combination of TGF-β1 if compared with sh control after 36 h of exposure (164 sh Cntrl) (***P-value <0.001-two-way ANOVA). (C) Crystal violet staining of WM164 empty vector control cells (164 EV), Twist1 overexpressing (164 Twist1) and Twist1 knockdown (164 sh Twist1) exposed to PLX4032 for 12 days (left). Quantification of the crystal violet staining was carried out at 590 nm by a microtiter plate reader (right).

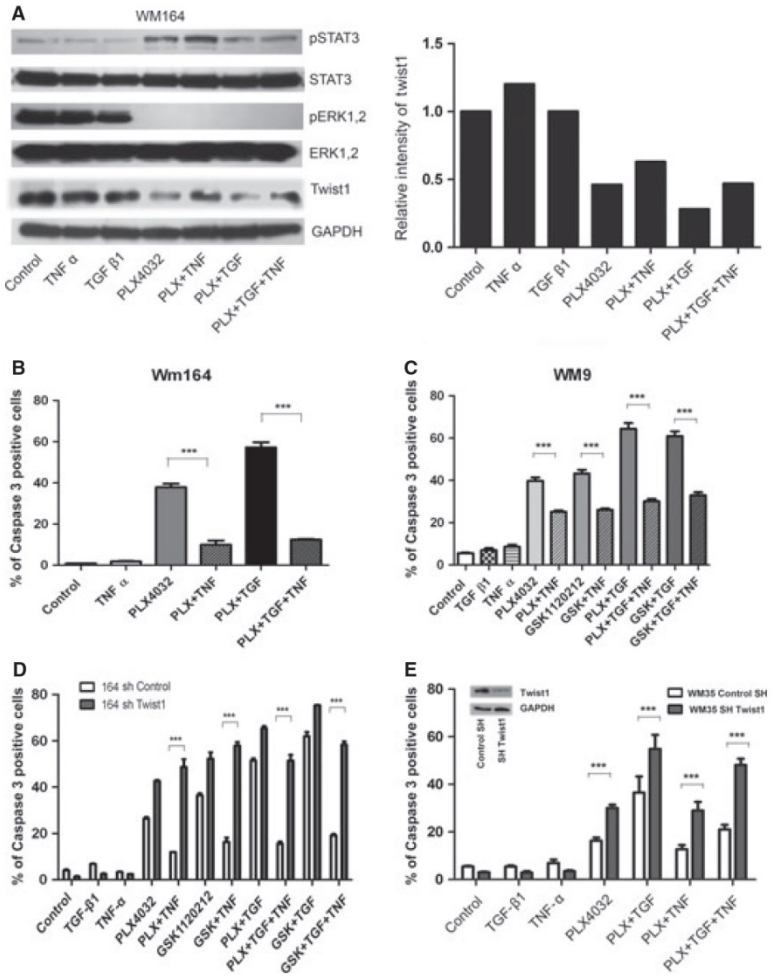

Figure 3. (A) Alterations of Twist1 levels in WM164 cells exposed to PLX4032 (1 μM), TGF-β1(5 ng/ml) and TNF-α (10 ng/ml) alone or in combination for 36 h (left), Twist1 bands were quantified and equalized to GAPDH as loading control (right). (B) TNF-α (10 ng/ml) induced rescue in WM164 cells exposed to PLX4032 (1 μM) for 36 h and also a combination of the same with TGF-β1 (5 ng/ml), depicted as percentage of caspase-3-positive cells (***P-value <0.001- Student's *t*-test). (C) TNF-α (10 ng/ml) induced rescue to PLX4032 (1 μM) and GSK1120212 (50 nM) for 36 h and a combination of the same with TGF-β1 (5 ng/ml) in WM9 (***P-value <0.001- Student's *t*-test). Comparison of TNF-α (10 ng/ml) induced rescue in Twist1 stable knockdown cells (164 sh Twist1) (D) and WM35 Twist1 stable knockdown cells (WM35 SH Twist1) (E) to control sh RNA (164 sh Control, WM35 SH Control)-transduced cells exposed to either PLX4032 or GSK1120212 in combination with TGF-β1 (5 ng/ml) for 36 h (***P-value <0.001- two-way ANOVA).

PLX4032 or GSK1120212. These findings were confirmed in other BRAF V600E–mutant cell lines WM9 and SKMEL28 (Figures 3C and S3D).

To dissect the effect of Twist1 expression in TNF-α-enhanced resistance, it was tested whether TNF-α could rescue the apoptosis-inducing effect of BRAF and MEK inhibitors in the absence of Twist1. To this purpose, WM164 sh-T along with WM35 stable Twist1 knockdown cells (WM35 sh-T) were employed. Exposure to TNF-α indeed rescued apoptosis induced by BRAF inhibitors in unspecific shRNA-transduced cells (164 shControl, WM35 ControlSH), whereas the rescue effect was significantly reduced in WM164 sh-T and WM35 sh-T cells (Figure 3D, E), suggesting that Twist1 is necessary for TNF-α-induced resistance against BRAF and MEK inhibitors. Similar results were obtained when the experiments were performed combining TGF-β1 with PLX4032 or GSK1120212 (Figure 3D, E).

Together, we have demonstrated that Twist1 as an antiapoptotic factor and single downstream target of multiple signalling cascades acts as a crucial regulator for resistance against BRAF and MEK inhibitors through a TGF-β1 and TNF-α axis. Previously, it has been reported that TNF-α induced resistance to BRAF inhibition through NF-kappaB signalling (Gray-Schopfer et al., 2007), which also induces Twist1 (Li et al., 2012). Our results further point to a differential regulation of Twist1 by TGF-β1 and TNF-α with a direct impact on cell survival towards a BRAF or MEK inhibition. More importantly, they suggest that TGF-β1 or TNF-α modulation of BRAF and MEK resistance could be strategically employed to new combinatorial therapeutic regimes. Additionally, overcoming Twist1 evoked loss of sensitivity to BRAF or MEK inhibition might lead to prolonged disease–free survival in patients.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Data S1. Materials and Methods.

Figure S1. (A) Relative cell viability of WM164 cells treated with PLX4032 (1 μM), GSK1120212 (50 nM) or in combination of either with TGF-β1 (5 ng/ml), in comparison with DMSO- (control) and TGF-β1-treated cells. (B) Percentage of caspase-3-positive cells after exposure to PLX4032 (1 μM), GSK1120212 (50 nM) in combination with TGF-β1 (5 ng/ml) for 36 h in WM9 cells (***P-value <0.001- Student's *t*-test). (C) Percentage of caspase -3-positive cells after exposure to SB431542 (10 μM), PLX4032 (1 μM) alone or in combination with TGF-β1 for 36 h in WM164 cells. (D) Western blot of Snail1 and Slug in WM164 cells treated with PLX4032 (1 μM) or TGF-β1 (5 ng/ml) alone, or in combination of both, with beta actin as a loading control.

Figure S2. (A) Twist1 overexpression (WM35 Twist1) in WM35 cells reduces the induction of apoptosis to treatment with PLX4032 (1 μM) even in the presence of TGF-β1 (5 ng/ml) if compared with empty vector control (WM35 EV) (***P-value <0.001- two-way ANOVA). (B) Immunoblot showing levels of Twist1 in WM164 EV, WM164 SH Twist1 and WM164 Twist1 overexpressing (WM164 Twist1 OV) cells before and after 12 days of PLX4032 (1 μM) treatment.

Figure S3. (A), (B) Percentage of caspase-3-positive cells in WM164 cells treated with TNF-α (10 ng/ml), TGF-β1 (5 ng/ml) and GSK1120212 (50 nM) alone or in combination in comparison with cells exposed to DMSO (control) (***P-value <0.001- Student's *t*-test). (C) Percentage of caspase-3-activated cells in WM164 cells treated with PLX4032 and the STAT3 inhibitor VII (1 μM) in comparison with a combination of the same with TNF-α (***P-value <0.001- Student's *t*-test). (D) Percentage of caspase-3-positive cells in SKMEL28 cells treated with TNF-α (10 ng/ml), TGF-β1 (5 ng/ml), PLX4032 (1 μM) or GSK1120212 (50 nM) alone or in combination (***P-value <0.001- Student's *t*-test).