Cell-autonomous and host-dependent CXCR4 signaling in cancer metastasis : insights from a zebrafish xenograft model

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Chapter 6

P53 stabilization by MDMX interference and CXCR4 receptor inhibition: a potential new strategy to attenuate Ewing sarcoma early metastatic events

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Abstract

The Ewing sarcoma family of tumors arises in children and young adults. Generally found in bones and soft tissues, these types of tumors share the EWSR1-ETS translocation, most commonly EWRSR1-FLI, which results in the formation of an oncogenic protein with aberrant transcriptional activity. Differently from most tumors, Ewing sarcoma is characterized by wild type P53, which makes the stabilization of this tumor suppressor a possible therapeutic approach to inhibit cancer progression. Here we propose MDMX, a negative regulator of P53, as a candidate drug target to limit Ewing sarcoma. Using a genetic approach, we show that MDMX inhibition partly affects Ewing sarcoma proliferation *in vitro* and *in vivo*. In addition, *MDMX* knock down increased mRNA levels of the chemokine receptor *CXCR4*, which is linked to metastatic Ewing sarcoma. Here we report that chemical and genetic inhibition of the cell autonomous CXCR4 signaling results in impaired Ewing sarcoma early metastasis formation in a zebrafish embryo model, where tumor cells are inoculated in the blood circulation. Moreover, reduced micrometastasis formation was also observed in a zebrafish mutant with a non-functional CXCR4 signaling. In conclusion, we propose MDMX and CXCR4 as targets for pharmaceutical intervention to inhibit Ewing sarcoma progression, affecting tumor burden and early metastasis formation, respectively. We suggest that the combined use of MDMX and CXCR4 inhibitors is a possible approach to combat Ewing sarcoma and further research is needed to investigate its efficacy in *in vitro* and *in vivo* models.

Introduction

Ewing sarcoma is an aggressive tumor of bones and soft tissues, mainly arising in children and young adults [1]. Histologically, Ewing sarcoma tumors are classified as small-round cell tumors and are often in a poorly differentiated state. The cell of origin is unknown and due to the possible onset of malignant transformations in different tissues, Ewing sarcoma is referred as Ewing sarcoma family of tumors [2]. The genetic signature shared by all tumors of the Ewing sarcoma family is the translocation *EWSR1-ETS*, coding for an aberrant transcription factor [3]. In 85% of the cases, *Fli1* is the member of the *ETS* transcription factor family that forms a chimeric protein together with *EWSR1*. In the remaining 15% of the cases, *EWSR1* forms oncogenic proteins with other transcription factors such as *ERG*, *ETV1* and 4 and *FEV* [4].

Although the genetic abnormality in the Ewing sarcoma family of tumors is known and represents an attractive molecular target for treatments, the main forms of current therapies for both localized tumors as well as metastatic disease are chemotherapy and radiotherapy [5]. Inhibiting angiogenesis, disrupting the interaction with the bone niche, thus preventing osteoclast induced bone resorption, as well as stimulating

antitumor immunity are potential therapies under experimentation to target the tumor microenvironment [5]. In addition, CXCR4 has been found to correlate with metastasis formation in Ewing sarcoma [6]. CXCR4, a seven-transmembrane G-protein coupled chemokine receptor, has been associated with tumor metastatic onset in secondary tissues expressing the cognate ligand, CXCL12 [7]. Hence, targeting CXCR4 is a possible approach to limit Ewing sarcoma progression. In addition to therapies designed to target the microenvironment, other pharmacological approaches have been proposed to limit the transcriptional activity of the fusion protein (YK-4-279) and limit tumor proliferation associated with CD99, IGF1R, PARP and PKCb signaling pathways [5]. Moreover, the stabilization of P53 represents another possibility for effective intervention [8]. Differently from the majority of tumors, the tumor suppressor P53 is often found in a wild type status in Ewing sarcoma tumors [9, 10]. Consequently, the stabilization of P53 results in the activation of cell cycle arrest, apoptotic mechanisms and senescence [11]. *In vivo* studies performed using a zebrafish model have recently demonstrated the promising effects of P53 stabilization on the inhibition of Ewing sarcoma cell proliferation [12].

Zebrafish is an increasingly popular animal model in cancer research and Ewing sarcoma has been modelled in both transgenic adult zebrafish as well as using xenografts of human cancer cell lines in embryos [12, 13]. We previously showed that Ewing sarcoma cell lines display different migratory properties and induce vascular remodeling once implanted in zebrafish embryos [12]. Moreover, in the same study, Ewing sarcoma proliferation was inhibited upon dual pharmacological treatment with Nutlin-3 [14] and YK-4-279 [15], inducing P53 stabilization and limiting the transcriptional activity of EWSR1-Fli1, respectively [12]. P53 transcriptional activation is regulated by MDM proteins, MDM2 and MDMX (also known as HDMX or MDM4) [16-19]. Differently from MDM2 that displays broader effects, MDMX regulates P53 in selective conditions [20]. Therefore, in parallel to MDM2 blockade by Nutlin-3, genetic targeting of MDMX represents a promising way to stabilize P53.

Interestingly, P53 is known to negatively regulate CXCR4 in breast tumors. Reactivation of P53 by Prima-1 inhibitor reduces tumor cell invasion and CXCR4 expression levels [21]. Because Prima-1 induces tumor cell apoptosis by reactivation of mutated P53, [22] , we investigated the effect of wild-type P53 reactivation on CXCR4 expression in Ewing tumors: we evaluated if observations in Ewing tumors could be compared to breast cancer or whether a combination between P53 stabilization and CXCR4 blockade is needed to impair Ewing sarcoma progression *in vivo*.

In this study we use a genetic approach to inhibit MDMX and stabilize P53 activity to assess the effect on Ewing sarcoma cell proliferation *in vitro* and *in vivo* and show that MDMX is a promising target for future therapies. Moreover, we found an MDMX-

dependent CXCR4 transcriptional regulation and demonstrate that chemical and genetic impairment of CXCR4 inhibits Ewing sarcoma cell proliferation *in vivo*. We propose that the combination of MDMX and CXCR4 blockade represents a potential line of intervention to limit Ewing Sarcoma progression.

Results

P53 stabilization via *MDMX* genetic interference in Ewing sarcoma cells reduces tumor cell survival *in vitro* and *in vivo*.

P53 stabilization was obtained by genetic inhibition of *MDMX*. Viral transduction of EW7 mCherry cells with targeted and doxocycline-induced shRNA against *MDMX* (shControl/i-*MDMX*) was performed and tumor proliferation was compared to the control condition (shCtrl/i-shCtrl). To assess whether *MDMX* knockdown (KD) affected Ewing sarcoma proliferation in a P53 dependent manner, the EW7 cell line was engineered with a *P53*-targeting shRNA or scrambled control (sh*P53*/i-sh*MDMX* or sh*P53*/i-shCtrl). First, the effect of P53 stabilization on tumor cell survival *in vitro* was assessed. EW7 cell proliferation was measured upon increasing concentrations of Doxocycline (2, 5, 10 and 20 ng/ml) to induce *MDMX* KD, by targeting the MDMX 3'UTR (i-shHx3') or the regulatory region (i-shHxR). Cells were treated for 120 hours. Cell survival was inhibited upon *MDMX* KD induction (using i-shHx3'or i-shHxR), in a dose dependent manner and P53 independent fashion (Figure 1A and B). Efficacy of *MDMX* KD induction was confirmed by Western blot, using 20 ng/ml Doxocycline for 72 hours (Figure 1C). Simultaneously, *P53* stable KD was also assessed (Figure 1C). Secondly, the effect of *P53* or *MDMX* KD on Ewing sarcoma proliferation *in vivo* was measured, using our zebrafish xenograft model. For this purpose the red fluorescent EW7 cell lines bearing a *P53*-targeting shRNA (sh*P53*/i-shCtrl), *MDMX*-targeting shRNA (shCtrl/i-sh*MDMX*) or a scrambled construct (shCtrl/i-shCtrl) were implanted in the yolk sac of 2 day post fertilization (dpf) zebrafish embryos with green fluorescent vasculature (*Tg(Fli1a:EGFP)*). For the *in vivo* experiment, i-shHx3' was used and from now on is referred to as i-sh*MDMX*. To induce *MDMX* KD, Doxocycline (20 ng/ml) was applied in the embryo water and refreshed every other day. No side-effects on embryo development were observed during treatment (data not shown). Tumor burden was quantified at 4 days after implantation (dpi), acquiring confocal fluorescence micrographs of each larva and performing image analysis in Image Pro, using a previously described macro to define the tumor proliferation readout as the number of objects multiplied by the mean object area. Genetic inhibition of *MDMX* resulted in reduced tumor burden, while no significant difference was observed between *P53* KD and control condition (Figure 2A, C, D, E). In order to verify if tumor burden inhibition upon *MDMX* KD was dependent on P53, EW7 sh*P53*/i-shCtrl, EW7 sh*P53*/i-sh*MDMX* and EW7 shCtrl/i-shCtrl were implanted in zebrafish embryos. Tumor burden was quantified as previously described

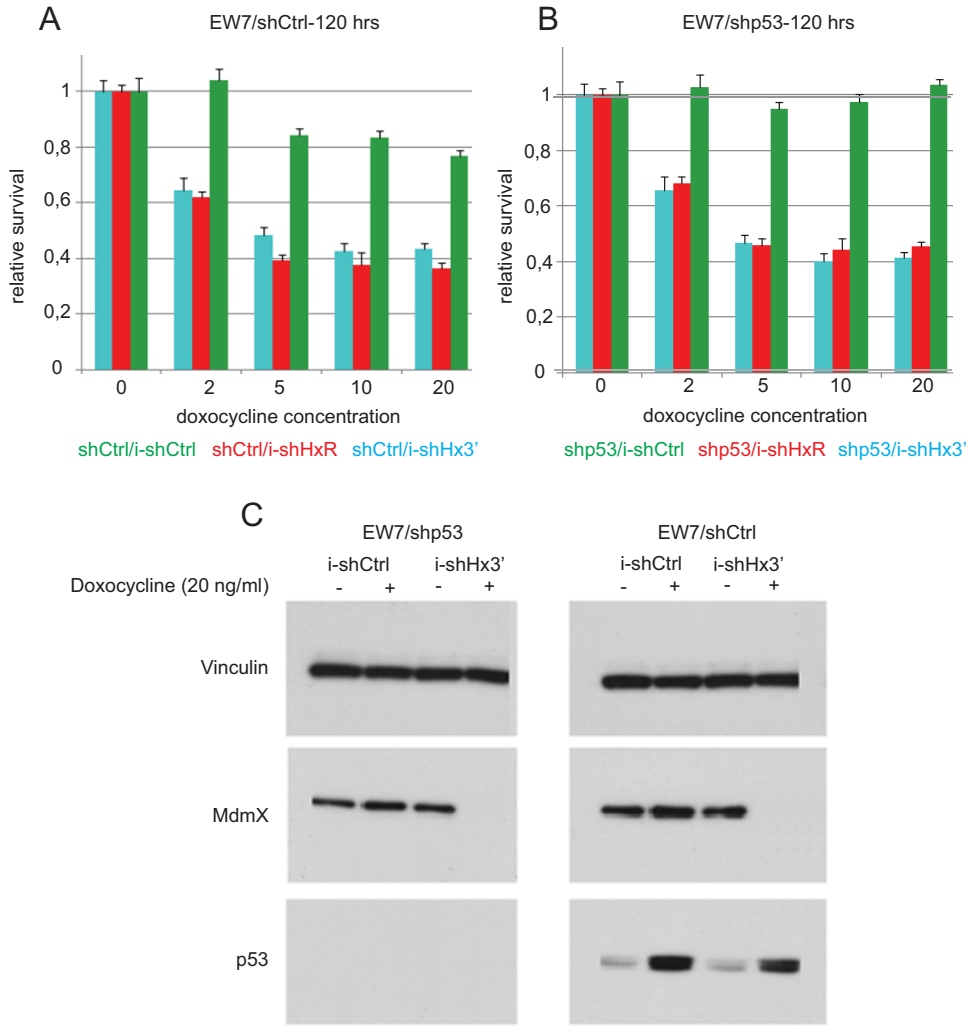

Figure 1. *MDMX* KD affects Ewing sarcoma cell survival *in vitro*. (A) *MDMX* KD effect (shCtrl/i-shHxR and shCtrl/i-shHx3') on cell survival was assessed in the EW7 cell line. Cell survival decreased upon increasing concentrations of doxocycline (2-20 ng/ml). Doxocycline concentrations equal to 5, 10 and 20 ng/ml led to similar cell survival reduction, compared to the control. (B) Cell survival was assessed upon *MDMX* and *P53* KD (sh*p53*/i-shHxR and sh*p53*/i-shHx3'). Reduced cell survival was found using increasing concentrations of Doxocycline (2-20 ng/ml). (C) Western blot confirms MDMX and P53 KD. Proteins were isolated from EW7-mCherry cells treated with 20 ng/ml doxocycline for 72 hours.

in 4 dpi larvae. No difference in tumor burden was detected when both *MDMX* and *P53* were knocked down, compared to the solely *P53* KD and control condition (Figure 2B, C, D, F). Taken together, our data show that *MDMX* KD affects Ewing sarcoma (EW7) cell survival *in vitro*, in a p53 independent manner, and inhibits tumor burden *in vivo* in a P53 dependent manner.

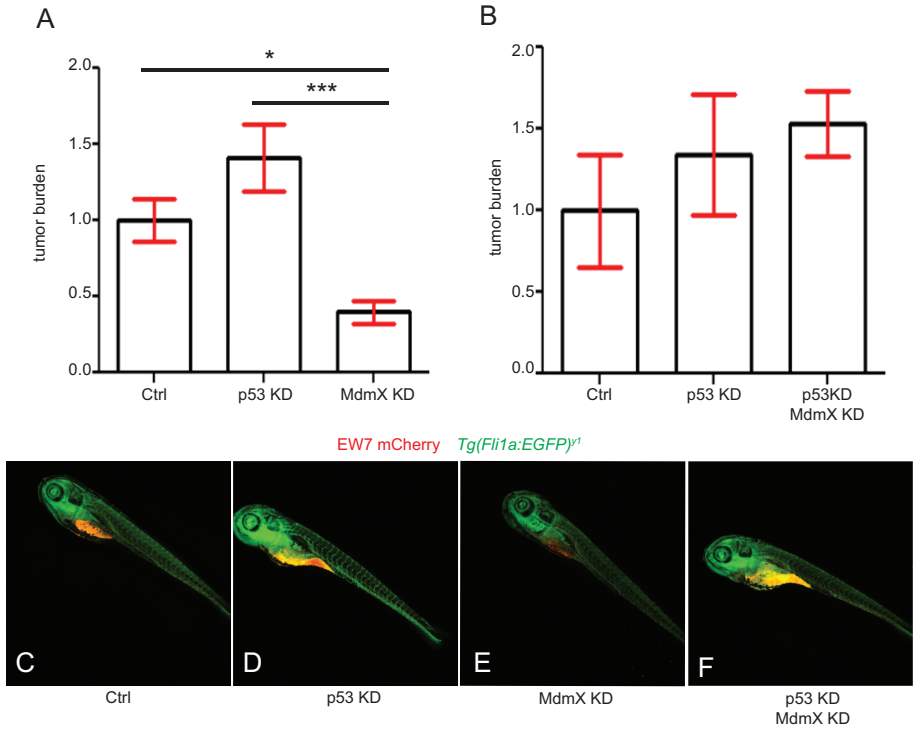

Figure 2. P53 stabilization via *MDMX* genetic knock down impairs Ewing Sarcoma burden *in vivo*. (A) EW7 mCherry tumor burden is compared in control (shCtrl/i-shCtrl), *P53* KD (sh*P53*/i-shCtrl) and *MDMX* KD (shCtrl/i-sh*MDMX*). A reduced tumor burden was found upon P53 stabilization (shCtrl/i-sh*MDMX*). *MDMX* KD induction was obtained via Doxocycline administration (20 ng/ml) in egg water. (B) EW7 mCherry tumor burden was compared in control (shCtrl/i-shCtrl), *P53* KD (shP53/i-shCtrl) and *P53* KD plus *MDMX* KD (shCtrl/i-sh*MDMX*). No difference in tumor burden was found when all conditions were compared. (C-F) Tumor burden is shown in control, *MDMX*, *P53* and *MDMX*/*P53* KD conditions in 4 dpi zebrafish larvae.

MDMX regulates *CXCR4* transcription in Ewing sarcoma cell line WE68

P53 acts as a negative regulator of CXCR4 and stabilization of the tumor suppressor has been found to correlate with reduced CXCR4-dependent metastasis formation in breast cancer [21]. To verify if P53 exerts the same regulation on CXCR4 in Ewing sarcoma, *CXCR4* mRNA levels were measured. RNA sequencing performed on different Ewing sarcoma lines revealed higher levels of *CXCR4* mRNA in WE68, compared to EW7 cell line, [23], previously used for the *in vitro* and yolk implantation experiments. Therefore,

CXCR4 expression was quantified in the WE68 td-tomato cell line, after induction of *MDMX* KD, in a *P53* WT or KD background, compared to control (sh*MDMX*/Wobble). Cells were treated with doxycycline (5 ng/ml) for 96 hours, before harvesting and RNA isolation. *CXCR4* mRNA levels, quantified by real time PCR, were found to be up-regulated in *MDMX* KD conditions, in a P53 independent fashion (Figure 3A). The efficiency of Doxocycline induced *MDMX* KD was confirmed, by measuring mRNA expression of *MDMX* (Figure 3B) and *P21* (Figure 3C). The P53 target gene *P21* was upregulated upon *MDMX* KD, as previously shown [24]. Moreover, P53 efficiency was also quantified, by measuring *P53* mRNA levels, via real time PCR (Figure 3D). In conclusion, a P53-dependent CXCR4 regulation is excluded in the Ewing sarcoma line WE68. *CXCR4* is negatively regulated by MDMX, making the use of CXCR4 targeting agents in parallel with MDMX-mediated P53 stabilization a possible approach to inhibit Ewing sarcoma proliferation and CXCR4-associated metastatic spreading.

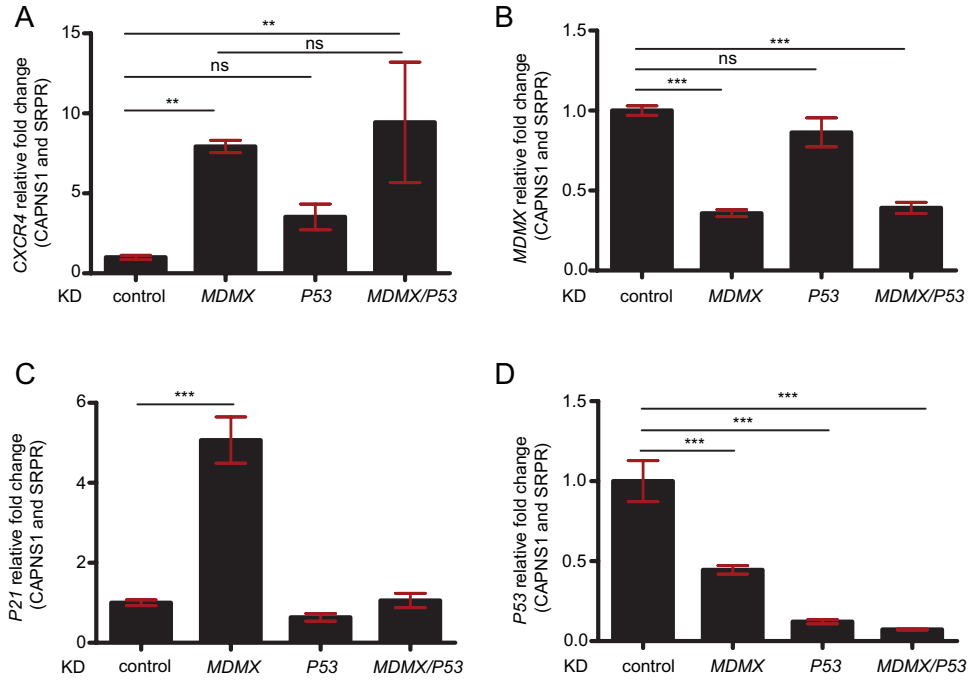

Figure 3. MDMX is a negative regulator of *CXCR4* expression. (A) *CXCR4* mRNA levels were checked upon *MDMX* and *P53* KD in the WE68 Ewing sarcoma cell line. Up-regulation of *CXCR4* expression was found in the *MDMX* KD background, independently from P53 stabilization. *MDMX* inducible KD was checked, measuring *MDMX* (B) and *P21* (C) mRNA levels. *P53* KD was confirmed (D). Statistical analysis was performed on 3 technical replicates (2 technical replicates for MDMX). One-way ANOVA. ***p<0.0001 or p=0.0005 (MDMX relative fold change), **p=0.002, ns p>0.05 (GraphPad Prism 5.0).

Ewing sarcoma cells inoculated in the blood circulation of zebrafish embryos induce vessel remodeling and initiate early metastatic events

We previously showed that *CXCR4* is upregulated upon *MDMX* KD, suggesting the importance of CXCR4 inhibition as additional treatment to P53 stabilization. As CXCR4 positively correlates with the metastatic dissemination of Ewing sarcoma [6], we investigated whether the WE68 cell line displayed a metastatic behavior in our *in vivo* model. For this purpose, WE68 td tomato cells were engrafted into the circulation of 2 dpf zebrafish embryos with green fluorescent blood vessels [*Tg(Kdrl:GFP)*^{s843}] (Figure 4A). Four days after engraftment, the WE68 phenotype was characterized by tumor induced angiogenesis at the site of implantation (duct of Cuvier) (Figure 4B and C) and by the initiation of early metastatic events. These events are characterized by tumor mass formation, distant from the injection site, in between blood vessels, in the area of the caudal hematopoietic tissue (CHT), functionally analogous to the fetal liver during mammalian development. The WE68 Ewing sarcoma cell line displays high levels of *CXCR4* mRNA and induced vascular remodeling and micrometastasis formation *in vivo*. Hence, blood inoculation of Ewing sarcoma cells in zebrafish embryos is a useful tool to study proliferation, angiogenesis and formation of micrometastasis in Ewing sarcoma, complementing previously described yolk xenograft techniques in zebrafish.

Chemical and genetic inhibition of CXCR4 signaling limits Ewing sarcoma early metastasis formation

CXCR4 correlates with metastatic events in Ewing sarcoma [6]. The WE68 cell line expresses high levels of *CXCR4* and forms micrometastasis in zebrafish embryos. We previously showed that interspecies cross talk is maintained between human and zebrafish, making the zebrafish xenograft model a valid *in vivo* model to study human tumors (Chapter 4). Using this model, we proposed a recently described CXCR4 antagonist (IT1t) to inhibit early metastatic events in triple negative breast cancer. Therefore, we took the same approach to verify whether blocking CXCR4 in Ewing sarcoma cells leads to tumor inhibition. WE68 cells were pretreated *in vitro* for 24 hours and then engrafted in zebrafish embryos through the duct of Cuvier, directly into the blood circulation. Tumor burden at the micrometastatic site was inhibited in 4 dpi zebrafish larvae engrafted with WE68 cells, with a chemically impaired CXCR4 signaling (Figure 5A-B). Importantly, we excluded possible side effects on tumor cells *in vitro*, due to the treatment with IT1t (20 μM). For this purpose, a trypan blue assay was performed after a 24 hour treatment and before engraftment. No difference in live cell percentage was found between IT1t and vehicle treated cells (Figure 5C-D).

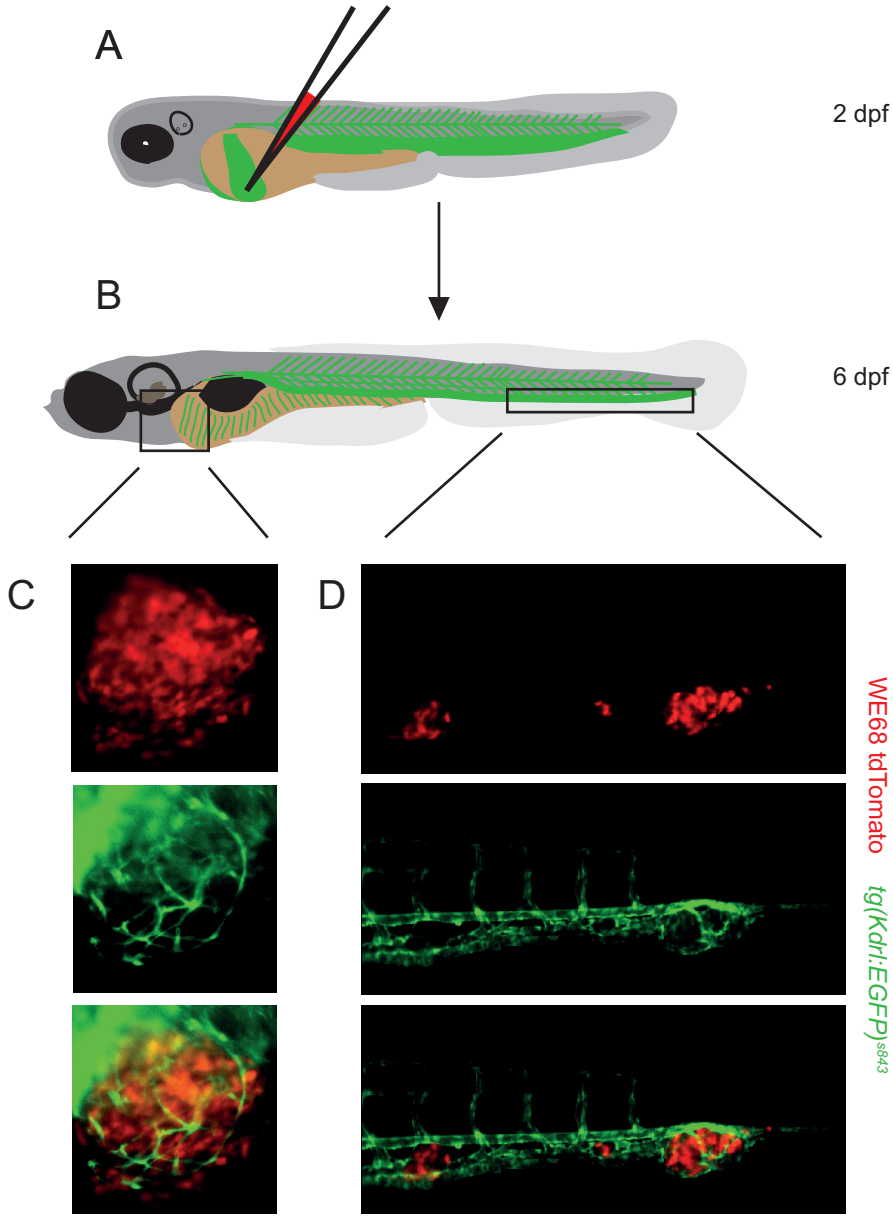

Figure 4. The Ewing sarcoma cell line WE68 induces angiogenesis and initiates early metastatic events in the zebrafish xenograft model. (A) Scheme of a 2 dpf zebrafish embryo and indication of engraftment site to inoculate Ewing sarcoma cells into the blood circulation are shown. (B) Scheme of a 4 dpi larva is shown, with enlargement of the areas where a primary (C) and secondary (D) tumor masses are formed. In (C), a complex tumor-induced vascular network is present.

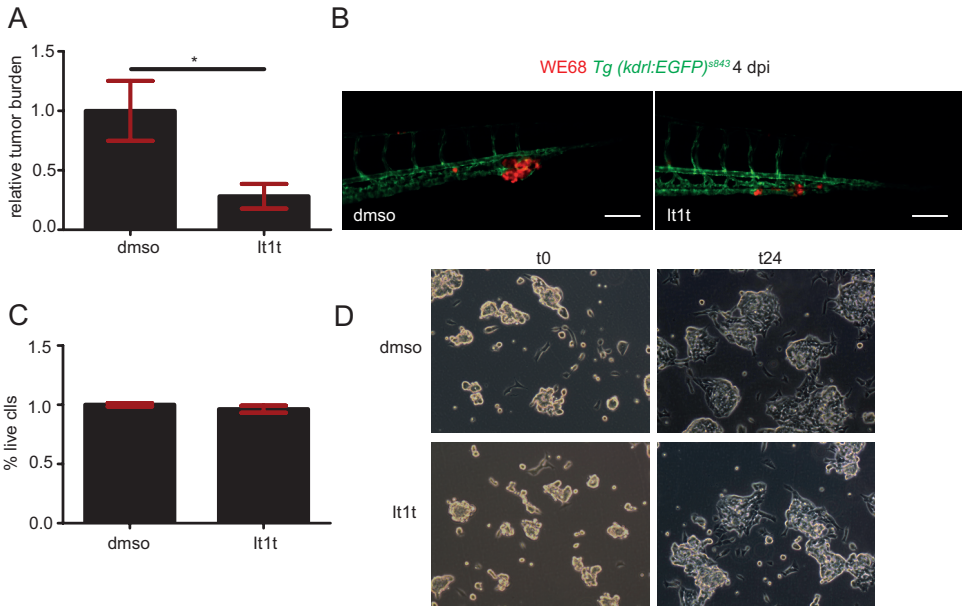

Figure 5. Chemical CXCR4 signaling inhibition on WE68 Ewing sarcoma cell line abolishes experimental micrometastasis formation *in vivo*. (A, B) Relative WE68 tumor cell burden in the CHT region of 4 dpi zebrafish larvae. Tumor cells were pre-treated in culture for 24 hours before engraftment. At 4 dpi, tumor burden was reduced (72% reduction) in the IT1t pre-treated group compared to the vehicle control. In (A) statistical significance is assessed with un-paired t-test and Welch's correction (1 replicate), * p=0.01. Dmso: n=27; IT1t: n=37. (C, D) IT1t effect on cell survival after a 24 hour incubation period is shown. No difference in percentage of live cells was found with a trypan blue assay. Data in (C) are mean±SEM of 4 measurements.

To confirm the effect of CXCR4 inhibition on Ewing sarcoma burden and early metastasis formation, the same experiment was repeated with another Ewing sarcoma cell line, characterized by high expression of *CXCR4* and ability to survive in the blood circulation and further develop micrometastasis in the zebrafish embryo model. While the WE68 cell line derives from a patient with a Ewing sarcoma of the fibula and pulmonary metastasis [25], TC32 originates from malignant cells in the ileum and adjacent soft tissues in a patient diagnosed with a primitive neuroectodermal tumor [26]. Both cell lines display high *CXCR4* expression, wt P53 and the EWSR1-Fli1 translocation [23]. TC-32 cells were pre-treated *in vitro* with IT1t (20 µM) for 24 hours and subsequently engrafted in the blood circulation of 2 dpf *Tg (kdrl:EGFP)^{s843}* zebrafish embryos. Tumor burden in the tail fin where micrometastasis occurred was reduced in 4 dpi zebrafish larvae (Figure 6A). After a 24 hour treatment *in vitro* with CXCR4 antagonist, TC-32 cell growth was not affected (Figure 6B). To confirm the effects of the pharmacological inhibition of CXCR4 in Ewing sarcoma cells, we used a genetic approach. *CXCR4*-targeting or a scrambled shRNA were delivered via viral transduction into TC-32 cells. A Ca^{2+} flux assay was performed to verify the functional impairment of CXCR4 signaling upon both chemical and genetic inhibition. TC-32 cells were pre-treated for 24 hours with 20 µM IT1t or vehicle (DMSO) and Ca^{2+} mobilization from intracellular storage

into the cytoplasm was measured after stimulation with 100 nM CXCL12. Increased fluorescent intensity was registered in the control condition after stimulation, while no difference was observed in cells subjected to CXCR4 inhibitor treatment. Similarly, Ca^{2+} mobilization was observed in TC-32 cells transduced with a control shRNA, while no changes in fluorescent intensity were observed in cells carrying a shRNA targeting *CXCR4*. In conclusion, both chemical and genetic CXCR4 impairment resulted in a non-functional CXCR4 signaling (Figure 6C). As additional validation, real-time PCR was performed and *CXCR4* mRNA levels were found to be down-regulated in TC-32 cells with genetic inhibition of CXCR4 compared to the control condition (Figure 6D). After validating the efficiency of the stable knock down, *CXCR4*-shRNA-TC32 and scramble-TC32 were injected into zebrafish embryos and metastatic tumor burden assessed in the tail fin at 4 dpi. Genetic inhibition of the CXCR4 signaling resulted in reduced burden and inhibited early metastatic events *in vivo* (Figure 6E and F). In conclusion, a reduced metastatic potential of Ewing sarcoma cells with impaired CXCR4 signaling *in vivo* makes CXCR4 a promising target for the pharmacologic treatments.

Host-dependent CXCR4 signaling influences Ewing sarcoma metastatic potential in a zebrafish xenograft model

The role of CXCR4 in tumor metastasis is extensively investigated and treatments to inhibit the chemokine signaling CXCR4-CXCL12 are under development to impair the ability of cancer cells expressing high levels of CXCR4 to make CXCL12-secreting tissues a favorable niche for growth [27]. The role of CXCR4 from the tumor microenvironment side is under investigation, as the interactions between tumor cells and stroma (endothelial and immune cells, fibroblast, cytokines and growth factors embedded in the extracellular matrix) are involved in supporting tumor progression [28]. To unravel the role of the stromal CXCR4 signaling in Ewing sarcoma progression, particularly focusing on the myeloid cell contribution, we engrafted both WE68 and TC32 cell lines into the blood circulation of a zebrafish host with an impaired CXCR4 signaling. As previously described, two *cxcr4* genes are present in zebrafish, *cxcr4a* and *cxcr4b*. As *cxcr4b* is highly expressed in neutrophils and macrophages [29] (Chapter 5), we used a *cxcr4b* homozygote mutant (*odysseus* or *ody*) [30]. Both WE68 and TC32 Ewing sarcoma cell lines displayed the inability to initiate early metastatic events and to form tumor aggregates at the hematopoietic site, where the formation of experimental micrometastasis takes place (Figure 7). In conclusion, the host dependent cxcr4 signaling is involved in the establishment of Ewing sarcoma micrometastases.

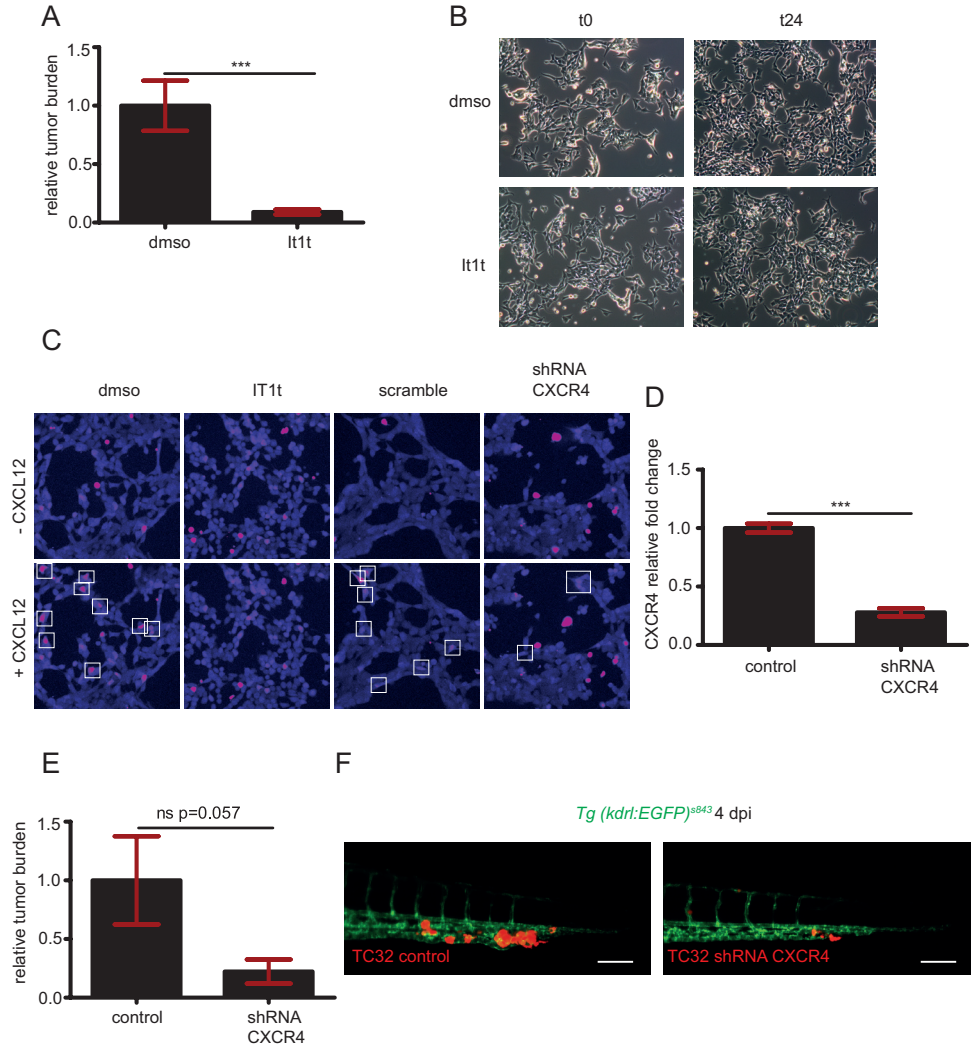

Figure 6. Chemical and genetic CXCR4 signaling impairment in TC-32 Ewing sarcoma cells recapitulate early metastatic event inhibition *in vivo*. (A) Relative tumor burden in dmso and IT1t at 4 dpi. TC-32 tumor cells were pre-treated in vitro for 24 hours with either IT1t (20 µM) or dmso control. Un-paired t-test with Welch's correction ***p=0.0002. Data are mean±SEM (1 experiment). Dmso: n=28; IT1t: n=21. (B) TC-32 cell line before and after IT1t (20 µM) and vehicle treatment. (C) Ca^{2+} flux was measured to verify the impairment of the downstream CXCR4 signaling upon chemical and genetic inhibition. TC32 cell line was treated with IT1t (20 µM) or dmso for 24 hours and stimulated with 100 nM CXCL12. Alternatively, the change in fluorescence intensity after ligand administration (100 nM CXCL12) was monitored in *CXCR4* stable knock down and scrambled control. Top and bottom panels show signal before and after ligand addition, respectively. Cells with increased signal intensity are indicated in the boxes. (D) Real-time PCR was performed to check *CXCR4* knock down efficiency. A72% reduction in *CXCR4* mRNA level was measured upon *CXCR4* knock down. (E, F) Tumor burden in the tail region is reduced (78%) in TC32 cells with *CXCR4* knock down, compared to scrambled control. Data in (E) are mean±SEM (1 experiment) and statistical significance is assessed with un-paired t-test. A trend towards tumor burden inhibition is found. Control: n=22; shRNA CXCR4: n=11. Scale bar in (F): 50 µm.

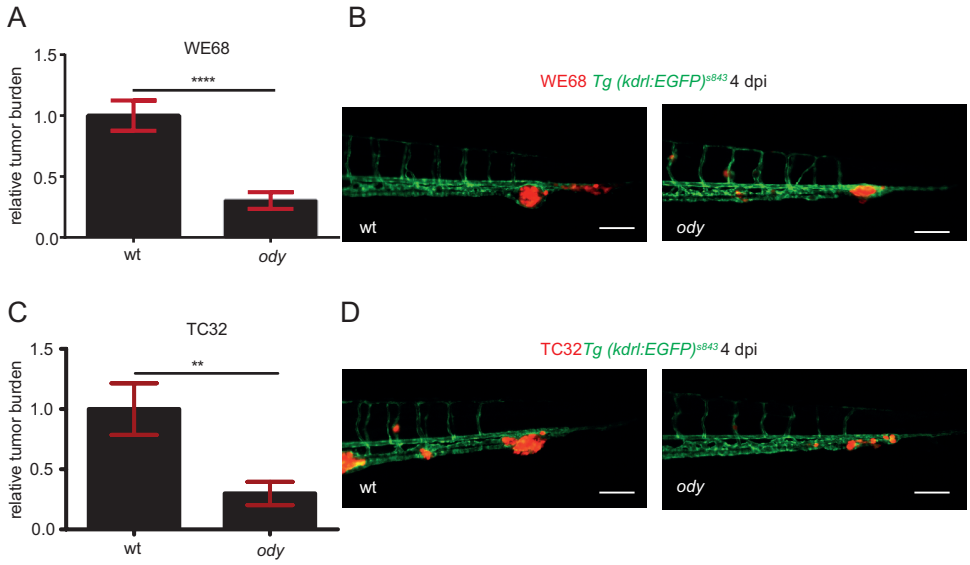

Figure 7. Cxcr4b-null zebrafish host limits the formation of early metastatic events by Ewing sarcoma cell lines. (A, C) Relative metastatic burden of the Ewing sarcoma lines WE-68 td-tomato and TC-32 td-tomato is affected in ody mutants compared to wt larvae at 4 dpi [70% reduction in tumor burden in the tail fin in (A) and (C)]. In (A) data are mean ± SEM of two independent experiments (wt: n=69, ody: n=39). Un-paired t-test, with Welch's correction ****p<0.0001. In (C) data are mean ± SEM (1 experiment) (wt: n=28, ody: n=17), un-paired t-test, with Welch's correction **p<0.005. (B, D) Ewing sarcoma cells form a compact and expanding tumor mass in the CHT region, between dorsal aorta and caudal vein. A reduced tumor cell aggregate is present in the ody mutant line at 4 dpi, for both WE68 and TC32 engrafted tumor cells. Scale bars: 50 µm. Micrographs are acquired using a Leica MZ16FA fluorescent microscope coupled to a DFC420C camera.

Discussion

The Ewing sarcoma family of tumors (ESFT) consists of Ewing sarcoma, peripheral primitive neuroectodermal and Askin's tumors. They all have in common the translocation *EWSR1-ETS*, caused by the rearrangement of the *EWS* gene on chromosome 22 with members of the *ETS* transcription factor family [31]. Although the genetic alteration that causes these types of tumors is known, still the current therapies are based on chemotherapy and radiotherapy. Interestingly, differently from many types of malignancies, P53, the so-called "guardian of the genome" [32], is often found in a wild-type status. Therefore, the stabilization of P53 represents a promising approach for the development of new therapies. Nutlin-3 blocks the interaction between P53 and MDM2, an E3 ubiquitin ligase that targets P53 for proteasome degradation, regulating in this way P53 availability and activity [33]. Work performed by our group demonstrated that Nutlin-3 treatment impairs Ewing sarcoma cell burden in a zebrafish embryo model. Moreover, the combinatorial treatment of Nutlin-3 and YK-4-279 that induces P53 reactivation, while blocking the transcriptional activity of the fusion protein EWSR1-ETS, synergistically inhibited tumor proliferation in the same *in vivo* model [12].

In tumors with WT P53, MDM2 and MDMX gain oncogenic functions [8, 20]. MDMX is a negative regulator of P53 [34]. MDMX binds the N-terminus of P53, impairing the transcriptional activity [8]. Accordingly, we propose that MDMX is a possible target for P53-stabilization-based therapies. Consequently, in order to investigate if MDMX has a role on Ewing sarcoma proliferation *in vivo*, we used the zebrafish xenograft model, that, together with transgenic models, has been demonstrated to be a powerful tool to study this type of sarcoma. Ewing sarcoma cells were engrafted in zebrafish embryos and tumor burden was found to be impaired upon induction of *MDMX* knock down, in line with *in vitro* data showing reduced cell survival. This suggests that MDMX could be a possible target for drug design and therapeutic intervention on Ewing sarcoma. In addition, the role of MDMX in controlling tumor growth *in vitro* (in a P53 dependent manner) and in a mouse model has been recently described in breast cancer [24]. Emerging compounds that inhibit MDMX have been recently described, such as SJ-172550, which interferes with MDMX binding to P53 and XI-100 that blocks MDMX transcription [8]. These drugs could be used in our in vivo model to phenocopy the effects of the genetic inhibition of MDMX on Ewing sarcoma proliferation.

At the time of diagnosis, about 25% of the patients present metastases [35]. In the case of pulmonary metastases, the event free survival post treatment is ~40%, while is <20% in the case of bone/bone marrow metastases [36]. Metastatic cancer is a major challenge for clinical research and strategies to tackle tumor spreading are constantly under development. High CXCR4 expression associates with metastases and poor patient survival in a Ewing sarcoma cohort analysis [6]. CXCR4 is a chemokine G-protein coupled receptor, controlling tumor cell migration and proliferation [37]. It drives tumor metastasis formation in organs that secrete the correspondent ligand, CXCL12 [38]. CXCR4 has been reported to be negatively regulated by P53 and CXCR4-mediated breast cancer metastases are reduced upon P53 activation [21]. We looked at CXCR4 expression upon P53 stabilization via *MDMX* KD and found that *CXCR4* is regulated by MDMX. This suggests the involvement of other homologues of P53, like P63 and P73, which, via the interaction with MDMX [39], might regulate *CXCR4* expression in Ewing sarcoma. Therefore, if MDMX-mediated P53 stabilization leads to increased *CXCR4* levels, the beneficial role of MDMX blockade on tumor proliferation might be overcome by metastatic event onset. We show that the inhibition of CXCR4 on the tumor cells side, either chemically or genetically, impairs experimental micrometastasis formation in a zebrafish xenograft model, where Ewing sarcoma cells are inoculated directly into the blood circulation. Finally, using the same *in vivo* model, we demonstrated that host-dependent CXCR4 mechanisms are involved in Ewing sarcoma early metastatic events, as tumor cells fail to form secondary cancer cell aggregates in a *cxcr4b* homozygote mutant. Therefore, CXCR4 represents a promising target for the development of new therapies to treat Ewing sarcoma. In conclusion, a combinatorial treatment to block both MDMX and CXCR4 might lead to an alternative therapeutic approach in metastatic Ewing sarcoma and requires further investigations.

Materials and Methods

Zebrafish husbandry

Zebrafish were handled according to the local animal welfare regulation and European directive (EU Animal Protection Directive 2010/63/EU). Zebrafish were maintained according to standard protocols (www.zfin.org). *Tg(fli1a:EGFP)* ^{y1}[40], *Tg(kdrl:EGFP)*^{s843} [41] and *cxcr4b* ^{t26035} [30] lines were used in this study.

Cell lines and RNA interference

Ewing sarcoma EW7-mCherry, WE68-td-tomato and TC-32-td-tomato cell lines were grown in gelatin (0.1%) coated flasks in IMDM medium complemented with 10% fetal calf serum (FCS), in a humidified atmosphere (5% CO2). G418 and Blasticidin (0.5 µg/ml) were used to select clones expressing mCherry and td-tomato, respectively. A third-generation lentiviral vector, FH1t with GFP tag [42] was harnessed to generate a shRNA targeting the 3'UTR region of MDMX in EW7-mCherry and WE-68 td tomato cell lines (sh*MDMX*: forward (Fwd) strand: 5'-TCCC ACAGTCCTTCAGCTATTTCAT TTCAAGAGA ATGAAATAGCTGAAGGACTGT TTTTTC 3' and reverse (Rev) strand: 5'-TCGAGAAAAA ACAGTCCTTCAGCTATTTCAT TCTCTTGAA ATGAAATAGCTGAAGGACTGT-3'). A shRNA targeting the regulatory region of *MDMX* (shHxR) was used in *in vitro* experiments. Most of the experimental procedures carried on in this chapter used the shRNA directed against the 3'UTR MDMX region (shHx3'). The shControl was directed toward mouse MDMX sequence, without knocking down the human MDMX (Fwd strand: 5'-TCCC GAATCTTGTTACATCAGCT TTCAAGAGA AGCTGATGTAACAAGATTC TTTTTC-3' and Rev strand: 5'-TCGAGAAAAA AGCTGATGTAACAAGATTC TCTCTTGAA ACATGGCTTCAAGAGATTC-3') in EW7 cell line, while a sh*MDMX*/Wobble control was used in WE68 cell line (Fwd strand: 5'-TCCC ACCGTCCGCAAGCTATGTCAT TTCAAGAGA ATGACATAGCTTGCGGACGGT TTTTTC-3' and Rev strand: 5'-TCGAGAAAAA ACCGTCCGCAAGCTATGTCAT TCTCTTGAA ATGACATAGCTTGCGGACGGT-3') [24]. Knock down induction was obtained using Doxocycline (5 ng/ml *in vitro* and 20 ng/ml *in vivo*). P53 stable knock down was obtained as previously described [43]. Cell lines containing knock down constructs were maintained under selection using Puromycin (1 µg/ml). All cell lines were provided by Dr. A.G. Jochemsen (LUMC). *CXCR4* stable knock down in TC32 cell lines was performed as previously described (Chapter 4 of this thesis).

Western blot

Western blot was performed to assess P53 and MDMX knock down efficiency. Cells were lysed and approximately 10-15 µg protein was loaded on gel. The following antibodies

were used: anti-P53 (DO-1/sc-126, 1:500; Santa Cruz Biotechnology®) and anti-HDMX (rabbit polyclonal A300-287A, 1:2000; Bethyl Laboratories®).

Cell survival assay

Cell proliferation *in vitro* was assessed using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega®).

***MDMX* KD induction *in vitro* and real-time PCR**

WE68 td-tomato cells were seeded in T25 flasks coated with gelatin (0.1%) to adhere over- night (O.N.). Doxocycline treatment (5 ng/ml) was performed for 96 hours to induce *MDMX* KD. After treatment, cells were harvested and RNA isolation, cDNA synthesis and real time PCR were performed to measure *CXCR4*, *MDMX*, *P53* and *P21* expression levels, as previously described (Chapter 4 of this thesis). The following primer sequences were used: *CXCR4* FW 5'-CAGCAGGTAGCAAAGTGACG-3' and *CXCR4* RV 5'-GTAGATGGTGGGCAGGAAGA-3'; *MDMX* FW 5'-AGGTGCGCAAGGTGAAATGT-3' and *MDMX* RV 5'- CCATATGCTGCTCCTGCTGAT-3'; *P53* FW 5'-CTCTCCCCAGCCAAAGAAGAA-3' and *P53* RV 5'-TCCAAGGCCTCATTCAGCTCT-3'; *P21* FW 5'-AGCAGAGGAAGACCATGTGGA-3' and *P21* RV 5'- AATCTGTCATGCTGGTCTGCC-3'; *CAPNS1* FW 5'-ATGGTTTTGGCATTGACACATG-3' and *CAPNS1* RV 5'- GCTTGCCTGTGGTGTCGC-3'; *SRPR* FW 5'-CATTGCTTTTGCACGTAACCAA-3' and *SRPR* RV 5'- ATTGTCTTGCATGCGGCC-3'. RNA isolation, cDNA synthesis and real time PCR were performed to measure *CXCR4* expression levels to verify knock down efficiency in TC-32 cell line.

Tumor cell suspension preparation and engraftment *in vivo*

Tumor cells were prepared for engraftment as previously described (Chapter 3 of this thesis). EW7-td-tomato cell lines were engrafted in the yolk sac of 2 dpf zebrafish embryos as previously described [12]. WE68- and TC32-td-tomato cell lines were inoculated in the blood circulation of 2 dpf zebrafish embryos, as previously described (Chapter 3 and 4 of this thesis).

Phenotype assessment

Tumor cell burden was assessed using Image-Pro Analyzer 7.0 (Media Cybernetics). Micrographs of single larvae (tail fin region) were acquired using a Leica MZ16FA fluorescent microscope coupled to a DFC420C camera. Single channels were overlaid in LAS AF Lite software and snapshots analyzed in Image-Pro Analyzer 7.0 (Media

Cybernetics). For each larva tumor burden was calculated based on number of objects multiplied by mean area and mean intensity, generated with a macro designed by H.de Bont (Toxicology, LACDR, Leiden University) and previously used to quantify tumor migration and proliferation [12, 44].

Statistics

Statistics analysis was performed using GraphPad 5.0. Un-paired t-test was used to compare data set of two groups. One-way ANOVA with Bonferroni *post hoc* test was used to compare dataset with three or more groups. Statistical significance was set at $p<0.05$.

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