The PAX-FOXO1s trigger fast trans-differentiation of chick embryonic neural cells into alveolar rhabdomyosarcoma with tissue invasive properties limited by S phase entry inhibition

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Abstract

The chromosome translocations generating PAX3-FOXO1 and PAX7-FOXO1 chimeric proteins paediatric fusion-positive alveolar subtype of Rhabdomyosarcoma (FP-RMS). Despite the ability to remodel chromatin landscapes and promote the expression of tumour driver genes, they only inefficiently promote the transformation of healthy cells into tumorigenic cells in vivo. The reason for this is unclear. To address this, we developed an in ovo model of neural progenitors to PAX-FOXO1s. Our data demonstrate that PAX-FOXO1s, but not wild-type PAX3/7, reduce the levels of CDK-CYCLIN activity and increase tissue invasion. The FOXO1s, similar to wild-type PAX3/7, reduce the levels of CDK-CYCLIN activity and increase tissue invasion. Introduction of CYCLIN D1 or MYCN overcomes this PAX-FOXO1-mediated cell cycle inhibition. Together, our findings reveal a mechanism that can explain the apparent limited oncogenicity of these factors. They are also consistent with certain clinical reports indicative of a neural origin of FP-RMS.

Author summary

The fusion-positive subtype of rhabdomyosarcoma (FP-RMS) is a rare malignant paediatric cancer that still remain to be deciphered. Out of the gross genetic aberrations found in these cancers, t(2:13) translocations are the first to appear and lead to the expression of fusion proteins made of the PAX or PAX7 and the transactivation domain of FOXO1. Both PAX3-FOXO1 and PAX7-FOXO1 have high transcription, yet they only inefficiently promote the transformation of healthy cells into tumorigenic cells in vivo. The FOXO1s, similar to wild-type PAX3/7, reduce the levels of CDK-CYCLIN activity and increase tissue invasion. Introduction of CYCLIN D1 or MYCN overcomes this PAX-FOXO1-mediated cell cycle inhibition. Together, our findings reveal a mechanism that can explain the apparent limited oncogenicity of these factors. They are also consistent with certain clinical reports indicative of a neural origin of FP-RMS.
oncoproteins, whose oncogenicity is limited by negative effects on cell cycle.

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Data Availability: Transcriptomes of ARMS and ERMS biopsies have been published elsew manuscript; accession numbers GSE92689, E-TABM-1202, E-MEXP-121). These are micro necessary to be able to compare data coming from distinct labs are also provided in S1 Table

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Introduction

Transcriptomic landscape remodelling represents a hallmark of tumourigenesis [1]. This is ofter of powerful transcriptional modulators, such as master transcription factors (TFs). Understandin lead to a pathogenic transformation of cells represents a key challenge in cancer research, so is more physiological model systems to address this question [1,2].

Two related oncogenic TFs, PAX3-FOXO1 and PAX7-FOXO1, are associated with the emerge paediatric alveolar subtype of rhabdomyosarcoma (RMS), named fusion-positive RMS (FP-RMS) patients are mostly found in limb extremities and the trunk. These comprise aggregates of round septa that express, as for other RMS subtypes, undifferentiated embryonic muscle cells marker almost half of FP-RMS patients carry detectable metastases in the lung or bone marrow at the these metastases, together with cancer resistance and emergence of secondary disease are to RMS patients [4].

The in-frame pathognomonic chromosomal translocations, t(2;13)(q35;q14) or t(1;13) (p36;q14) PAX7 genes to the 3' end of the FOXO1 gene and lead to the mis-expression of chimeric TFs m PAX3 or PAX7 TFs and the transactivation domain of FOXO1 [3]. Exome sequencing revealed t primary genetic lesions in more than 90% of FP-RMS cases [5,6]. Few somatic mutations are f relative fast development of the tumour after the translocations [6]. Furthermore, recurrent gros
whole genome duplication, unbalanced chromosomal copy gain, focal amplifications (12q13-q14) and heterozygosity notably on 11p15.5 locus presented by FP-RMS cells [5,6] suggest a tumorigenic chromothripsis [7]. The relative contribution of PAX-FOXO1s and of these gross genetic aberrations to FP-RMS cells is still debated.

A large body of work, mainly focused on PAX3-FOXO1 and aimed at identifying and functionallytarget genes, argues the cell fate change characteristic of FP-RMS is driven by PAX-FOXO1s [8] from PAX-FOXO1’s strong transcriptional transactivation potential, which surpasses that of normal FOXO1. FOXO1 binds to non-coding cis-regulatory genomic modules (CRMs), remodelling chromatin around these CRMs regulate the expression of genes associated with at least 3 traits deleterious to FP-RMS [8,9,11,12]. First, several of the target genes encode cell surface proteins which are key cell alteration of the some of them was shown to affect RMS cell motility [15–19]. Second, FP-RMS muscle cell master TFs, which in presence of PAX3-FOXO1 can no longer promote muscle terminal differentiation, PAX-FOXO1s perturb the core cell cycle machinery [8,9]. Cross-interactions between PAX3-FOXO1 and BCL-XL or the senescent factor p16INK4A promote cell survival [22–24]. PAX3-FOXO1 increase myoblasts and this associated with a downregulation of cyclin-dependent kinase inhibitors (CDKIs) [8].

Despite the apparently powerful activity of PAX-FOXO1s, data from animal models have led to the conclusion that these proteins do not efficiently trigger FP-RMS formation and spreading [24,28–31]. In excess of 60% of PAX3-FOXO1 expressing human myoblasts or mesenchymal stem cells to produce significant FP-RMS contrasts with the 15 days required for patient derived FP-RMS cells [30–32]. Similarly, driving PAX3-FOXO1 in muscle embryonic cells from the murine Pax3 locus induces tumour mass with a reported frequency of 60%, these in vivo approaches have revealed several parameters enhancing PAX-FOXO1 proteins or transgenes in zebrafish indicated that neural derived tissues are more prone than mesodermal when exposed to PAX3-FOXO1, highlighting the differential response of distinct cell lineages [2]. These models both indicate that a threshold level of PAX3-FOXO1 needs to be reached to observe tumour formation complementing PAX-FOXO1s expression with genetic aberrations promoting cell cycle progression increased the frequency of tumour formation [28–34]. This was notably achieved by lowering the retinoblastoma protein, RB1; or conversely by ectopically elevating MYCN expression or RAS a fusion protein displays elevated levels in the G2 phase which are required for the upregulation of target genes [27].

To investigate the molecular mechanisms of oncogenicity in FP-RMS we characterised the initiation of tumours associated with the transformation of cells expressing PAX3-FOXO1 and PAX7-FOXO1. The genetic origin of paediatric cancers [35], the identification of FP-RMS growths in neural tube derived tissues of embryonic neural lineage determinants in FP-RMS cells [9], and the recent use of chick embryonic neural tube progenitors for cell invasion [38,39] led us to develop the embryonic chick neural tube as a model system. We repress the molecular hallmarks of neural tube progenitors within 48 hours and impose a molecular definition of FP-RMS cells. Concomitantly, PAX-FOXO1s promote an epithelial-mesenchymal transition, correlate the adjacent mesoderm in less than 72 hours. Moreover, PAX-FOXO1s limit cell cycle progression activity, which in turn can explain the limited oncogenicity of these fusion TFs.

Results

Chick neural cells lose their neurogenic potential upon PAX3-FOXO1 exposure

To investigate the transformation potential of PAX-FOXO1 proteins, we set out to perform gain of function in the neural tube of chick embryos. Hamburger and Hamilton (HH) stage 11 chick embryos were electroporated with PAX3-FOXO1 together with a bi-cistronically encoded nuclear-targeted GFP and all (Fig.1A). For comparison, electroporations with the wild-type versions of Pax3 or the empty pCIC

https://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1007037
addition, the non-electroporated side of the neural tube stood as well as an internal control.

![Figure 1](https://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.10...)

**Fig 1. PAX3-FOXO1 switches off generic neurogenic marker expression in chick embryonic spinal cord.**

(A) Schematics showing HH10-11 chick embryos filled with pCIG based constructs before being dissected 12 to 72 hours post-electroporation (hpe) to thoracic region (dark blue square) of the electroporated embryos were dissected 12 to 72 hours post-electroporation (hpe) for FACS based analyses. (B) (i-ii”; iv-v””) Immunodetection of GFP, SOX2 and HUC/D on transverse sections of electroporated embryos 48hpe with the indicated plasmids. MZ: Mantle Zone; SVZ: Sub-Ventricular Zone. (i) HUC/D+ cells in the GFP+ population 48hpe with the indicated plasmids (dots: embryo value Whitney U test: *: p<0.05, ***: p<0.001, ****: p<0.0001). (C, D) Immunodetection of GFP, PA and HUC/D sections of chick embryos 48hpe with the indicated plasmids. DRG: dorsal root ganglia. Arrows point at SOX2 or PAX6. hpe: hours post-electroporation; scale bars: 50μm.

We characterised the molecular identity of electroporated cells by assaying the expression of generic neurogenic marker expression. At 48 hours post-electroporation (hpe), the neural tube of chick embryos contained SOX2+ cells to the ventricle and HUC/D+ neurons laterally in the mantle zone (brackets in the non-electroporated side). PAX3 overexpression did not affect this organisation and cells kept expressing these markers (Fig 1Bi-i”,iii,iv-iv”,vi). This is consistent with PAX3 being present in the spinal progenitors located in the dorsal part of the neural tube. Yet, in some cells expressing high levels of PAX3, SOX2 and PAX6 expression levels were diminished (Fig 1Ci”). More significantly, spinal cells overexpressing PAX3 produced less HUC/D+ neurons and PAX6, while those in iv’, iv” point at a HUC/D+; GFP+ cell. Arrows in Bi’,i” and Ci”,” mark GFP+ SOX2 or PAX6. hpe: hours post-electroporation; scale bars: 50μm.

We next checked for the expression of SOX10, a marker of neural crest cells (NCC) ([41](https://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.10...)). At 48hpe SOX10+ NCC were present in the skin and the DRG (insets in Fig 1Di,ii). The electroporation induced SOX10 expression (Fig 1Di-i”) and only rare PAX3-FOXO1+ cells were positive for this marker. This rules out the possibility of a switch of neural cells into NCC upon exposure to the fusion TF.
PAX3-FOXO1 is sufficient to divert cells from a generic neurogenic program.

PAX3-FOXO1 TFs convert chick neural cells into FP-RMS like-cells

We next tested whether PAX3-FOXO1 expressing cells adopted the identity of alveolar rhabdomyosarcoma (RMS) cells. To identify genes that define this identity [9], we combined and re-analysed microarray-based tumour tissue data from 34 PAX3-FOXO1 positive FP-RMS patients and 59 patients affected by other RMS subtypes (Tables, S1A Fig) [42–46]. We identified 1194 genes enriched in FP-RMS biopsies; 40% of which identified PAX3-FOXO1 bound cis-regulatory modules (CRM) [11,12] (Fig 2B). This list of genes included PAX3-FOXO1 dependent FP-RMS markers, such as ALK, ARHGAP25, or FGFR4 [9].

Other genes indicated that they encode for developmental regulators of many embryonic lineages known to possess PAX7 activities (Fig 2C, S4 Table) [48], and not exclusively of the muscle lineage. For instance, the caudal part of amniotes, ALK is found in the spinal cord neurons and peripheral nervous system expressed by the neural tube and somite cells (cf. chicken expression database http://geisha.arbimarks amongst others the somites [50]. The complexity of FP-RMS signature is likely to stem from the expression of master TFs which control the development of distinct lineages in the embryo and during different developmental time points. To illustrate this, we focused on nine TFs, namely EYA2, FOXF1, LMO4, PAX2, PRDM12 and TFAP2β (Fig 2D). In the myogenic lineage, MEOX1 is the first to be induced and has a crucial role in specification and the segmentation of the epithelialized somites [51]. LMO4 is transiently induced and remains longer in these structures where it contributes to the induction of one of the core myogenic regulators, PITX2 has been shown also to contribute to the induction of MYOD1 but in the limb myoblasts [52]. Only briefly expressed in the somites, FOXF1 marks the boundaries of the splanchnic mesoderm or the sclerotome [57]. The other TFs, PAX2 [58], PRDM12, PITX2, and LMO4 are expressed by the peripheral and/or central nervous system, and so are EYA2 [61], LMO4 [62] and PITX2. These cells are not simply undifferentiated muscle cells, but rather as cells with their own transcriptional programs.

In this combination of TFs in FP-RMS, we quantified their expression levels using either RT-qPCR and established human RMS cell lines, including 3 FN-RMS (RD, RDABl, Rh36) and 4 PAX3-FOXO1 (SJRh30, Rh4, Rh5) (Fig 2D, S1B Fig, S1 Methods, S1–S4 Raw images). All markers assessed with transcript and protein expression levels varying from one cell line to another (Fig 2D, S1B i, ii, iii). PRDM12 and TFAP2β displayed significant elevated levels in FP-RMS cells compared to FN-RMS, whereas MYOD1 protein levels did not discriminate FN-RMS and FP-RMS cell lines (S1Biii Fig). FOXF1 and LMO4 transcripts were detected in all RMS subtypes (Fig 2Dv). In higher in FP-RMS Rh5, SJRh30 and Rh4, cell lines than in the other cell lines (S1Biii Fig), representing a biomarker and supporting post-translation regulation. Altogether these results further highlight the combination of TFs expressed by FP-RMS [64], which could in turn underpin the transcriptomic evidence of the identity of the nine TFs chosen can be used to define a FP-RMS identity and to discern embryonic lineage, notably the myogenic one.
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molecular signature reminiscent of human FP-RMS cells in neural cells, a non-muscle lineage. MYOD1 was induced by PAX3-FOXO1, another member of the core myogenic transcriptional network used as a RMS marker [64], was not induced by the fusion TF, nor by PAX3 (S2B Fig).

**Fig 3.** PAX3-FOXO1 converts embryonic neural progenitors into cells harbouring FP-RMS molecular traits.

(A) mRNA expression levels of FP-RMS hallmark genes in GFP+ FACS sorted neural tube cells from pCIG plasmids. Levels are relative to TBP transcripts and normalised to pCIG samples mean level bar plots: mean ± s.e.m; n> 4 FACS sorts; v: heatmap exhibits mean value over 4 discrete FACS samples are colour-coded in blue (lower levels) to yellow (higher levels). (B) *In situ* hybridisation of MYOD1 detection and immuno-detection of GFP on transverse sections of chick embryos 24 hrs after electroporation. x and x' panels represent the same neural tube but in adjacent histological slides. x' region of the x' sample. dm: dermo-myotome; DRG: dorsal root ganglia, myo: myotome. Upper negative for MYOD1 (**), the lower one cells positive for this TF. PITX2a, LMO4: n>9 embryos; ii**) Immunodetection of GFP and TFAP2α on transverse sections of chick embryos 48hpe w Quantification of expression levels of TFAP2α in GFP+ cells in the spinal cords of chick embryos (dots: cell values; bars: mean ± s.e.m; n>5 embryos). Mann-Whitney U test p-value between either pCIG and Pax3 samples or pCIG and PAX3-FOXO1 samples: *: p<0.05, **: p<0.0001, ns: p>0.05; Scale bars: 50μm.

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**PAX3-FOXO1 activates conserved FP-RMS associated enhancers in chick neural cells**

The robustness of PAX3-FOXO1 mediated FP-RMS hallmark gene induction in neural cells could be confirmed by the presence of conserved enhancers known to operate in FP-RMS cells [11,12]. To test this idea, we cloned each mouse *Met, Meox1, Myod1, Alk*, or human *CDH3* and *PRDM12* genes (S1 Methods). We cloned each promoter and a reporter gene and co-electroporated them with either pCIG, Pax3, or PAX3-FOXO1. The activity of these enhancers was barely detectable (Fig S3) near the *PRDM12* locus that had an endogenous activity in the intermediate-dorsal neural tube, presence of PAX3-FOXO1 all cloned enhancers, except the *CDH3* CRM, were transcriptionally active.
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Fig 5. PAX3-FOXO1 transforms neural epithelial cells into a cohesive mesenchyme capable of migration. 

(A) (i-iii') Immunodetection of GFP and DAPI staining on transverse section of chick embryo plasmids. Brackets in Aiii highlight cells delaminating inside the neural tube (in) or outside of Apical confocal views in open-booked preparation of spinal cords of embryos 48hpe with the immunolabelled with antibodies against GFP and PARD3. Variations in the phenotype are ob FOXO1: (iii) represents 2 of 8 cases analysed, (iv) the rest of cases. (v-vi') Immunodetection transverse sections of chick embryos 48hpe with the indicated plasmids. (C) (i-iii') Z-projecti embryos immuno-labelled for GFP and stained with DRAQ5. Dotted lines delineate either the neural tube/mesoderm border. (iv) Quantification of the distance of each GFP nuclei from the indicated plasmids (Violin plots) P: progenitors and N: neurons. (D) (i) Exponential fit of function of cluster size at 48hpe in discrete sample types. (ii-iii') Immunodetection of GFP ar sections of chick embryos 48hpe with the indicated plasmids. (E) Gene ontology enrichment linked cell migration and adhesion applied to genes enriched in FP-RMS biopsies. EMT: epithelial transition to mesenchymal transition. (F) (i-ii) Levels of mRNA expression of the indicated assayed by RT-qPCR on GFP + FACS sorted neural tube cells 48hpe with pCIG, Pax3 and P to TBP transcripts and normalised to pCIG samples mean level (dots: value for a single RNA Whitney U test p-value: *: p<0.05, **: p<0.01, ns: p>0.05). x' and x" panels are blow-ups of a basal lamina. Scale bars: 50μm, but in D: 10μm.

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To validate these observations, we quantified several parameters in whole embryos stained with and documented the distribution of several key markers of the epithelial state (S3A Fig, S6 Table) migration are tightly connected to cell shape (e.g. [65]). Hence, we started by evaluating that of ellipticity of their nuclei segmented from 3D images (S3B Fig). This parameter reflects the degree fluctuated between 0.4 and 0.42 for pCIG and Pax3 elongated nuclei (S3Bi,ii,iv Fig). The elliptic substantially smaller; with time this difference was accentuated (S3Biii,iv Fig). PAX3-FOXO1 ce adapted to tissue exploration [65].

We then monitored the orientation of the major axis of the ellipsoid fit of GFP + cells using polar of cell arrangement within the tissue (S3C Fig). The polar angle θ gave the deviation of the nuclear ventral axis, while the azimuthal angle φ informed on its orientation within the lateral-medial axis (S3Ci Fig). In 48hpe controls and Pax3 samples, the distribution of θ and φ was similar (S3Cii,ii 0°C, consistent with nuclei parallelising the medial-lateral axis of the embryos and apico-basal atl...
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Fig 6. Pax3 and PAX3-FOXO1 limit the entry of cells into S phase.

(A) (i-ii') Immunodetection of GFP, phospho-histone H3 (PH3) and DAPI staining on transverse sections of chick embryos 48hpe with the indicated plasmids. (iii) Quantification of the number of PH3+ cells in the GFF expressing the indicated plasmids (dots: embryo values; bar plots: mean ± s.e.m.).

(B) (i-i'') and DAPI staining on transverse sections of chick embryos 48hpe with the indicated plasmids before harvest. (iii) Quantification of the number of PH3+ cells in the GFPl expressing the indicated plasmids (dots: embryo values; bar plots: mean ± s.e.m.).

(C)(i) FACS plots showing DNA content of neural cells stained with vybrant dyecycle violet stain and cell cycle phases gating (green: G0, pink: G2/M phase). (ii-iv) Percentage of cells in the indicated cell cycle phase at 48hpe estimated from the FACS plots, for individual values see S6Bi-iii Fig and for raw plots see S6A Fig.

(D) (i-i'') Immunodetection of GFPl and DAPI staining on transverse sections of chick embryos 48hpe with the indicated plasmids. P: progenitors; N: neurons. (iii) Quantification of pRB1 levels in the GFP+ cells in embryos expressing the indicated plasmids. P: progenitors; N: neurons. (dot s.e.m., n=4 embryos). x' and x'' panels are blow-ups of a subset of x panel GFP+ cells. Mann-Whitney U test: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001, ns: p>0.05. Scale bars: 50μm.

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To assess the proliferative state of cells, we marked mitotic cells using an antibody against the phosphorylated form of RB1 (pRB1) and DAPI staining on transverse sections of chick embryos expressing the indicated plasmids. P: progenitors; N: neurons. (dot s.e.m., n=8 embryos). (E) (i-ii') Immunodetection of GFPl sections of chick embryos 48hpe with the indicated plasmids. (ii) FACS plots showing DNA content of neural cells stained with vybrant dyecycle violet stain and cell cycle phases gating (green: G0, pink: G2/M phase). (iii) Quantification of pRB1 levels in the GFP+ cells in embryos expressing the indicated plasmids. P: progenitors; N: neurons. (dot s.e.m., n=4 embryos). x' and x'' panels are blow-ups of a subset of x panel GFP+ cells. Mann-Whitney U test: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001, ns: p>0.05. Scale bars: 50μm.

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phase. Similar experiments performed in human fibroblasts indicated that this cell type was also FOXO1 (S6Biv-vi Fig), supporting the idea that PAX3-FOXO1 mediated cell cycle hold is not in

Finally, the phosphorylation of the retinoblastoma-associated RB1 protein being one of the hallmarks leading to the entry in S phase, we assayed its status (Fig 6D). Both PAX3 and PAX3-FOXO1 fusion protein to a greater extent than wild-type PAX3 (Fig 6D). Yet, it is worth noting that phospho-cells overexpressing the PAX variants and were higher than cells that have left the cell cycle, so hence, cells are probably not fully arrested. The decrease in phospho-RB1 levels is not linked to RB1, CDK2, CDK6 and CCND1 (S5C Fig). Instead, we identified that amongst the CIP/KIP CDKs upregulated by the fusion protein (Fig 6E), a cue potentially explaining the PAX3-FOXO1 media activity.

PAX3-FOXO1 mediated cell cycle inhibition is overcome by CCND1 or MYCN

We then wanted to test whether PAX3-FOXO1-transformed cells could re-enter cell cycle. For this, we tested the CDK-CYCLIN activity in PAX3-FOXO1 expressing cells, by forced expression of CYCLIN D1, a cyclin subtype. PAX3 and PAX3-FOXO1 positive cells displayed a mitotic rate, revealed by quantification of the number of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-positive nuclei and the apical surface at 72hpe with the indicated plasmids (Violin plots). (D) PITX2 expression on transverse sections of chick embryos 24hpe and 48hpe with the indicated plasmids and in adjacent section slide. Bottom panels show areas on the embryos and section presented in t

The PAX-FOXO1s trigger fast trans-differentiation of chick embryonic neural tube. A recent study indicated that PAX3 and PAX3-FOXO1 mediate the suppression of cell cycle in chick neural tube. To understand the biological significance of this inhibition, we investigated whether PAX3-FOXO1-transformed cells could re-enter cell cycle. For this, we tested the CDK-CYCLIN activity in PAX3-FOXO1 expressing cells, by forced expression of CYCLIN D1, a cyclin subtype. PAX3 and PAX3-FOXO1 positive cells displayed a mitotic rate, revealed by quantification of the number of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-positive nuclei and the apical surface at 72hpe with the indicated plasmids (Violin plots). (D) PITX2 expression on transverse sections of chick embryos 24hpe and 48hpe with the indicated plasmids and in adjacent section slide. Bottom panels show areas on the embryos and section presented in t

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We next wondered whether the proto-oncogenes recurrently amplified in FP-RMS cells could also appear in FOXO1 expressing cells. MYCN has been amplified in about 10% of FP-RMS [6], we forced its expression in the control neural tube, as previously demonstrated [68]. MYCN, as opposed to its usual function in neural progenitors in M phase (Fig 7Aiii). In contrast, in presence of PAX3-FOXO1 and MYCN cells b (Figs 7Aiii and S5D), with their rate of mitosis reaching levels comparable to that of control pCIC

Finally, we checked that upon reactivation of the proliferative activity of PAX3-FOXO1, the specific neural progenitors were maintained. Assaying the migration of cells supported this idea (Fig 7C) marker gene PITX2 (Fig 7D).

Together, these results indicated that PAX3-FOXO1 proteins inhibit the entry of cells into S phase and decrease in CDK-CYCLIN activity. This inhibition can be overcome by increasing the levels of CI

PAX7-FOXO1 transformation of spinal progenitors is reminiscent to that by PAX3-FOXO1

Finally, we assessed whether the transformation properties of PAX3-FOXO1 were shared by PAX7-FOXO1. The effects of PAX7-FOXO1 on spinal progenitors diverge from that of PAX7 (Fig 8, S7 Fig). To do so, we assayed cells 48hpe with PAX7-FOXO1 or Pax7 using the pan-neuronal markers SOX2 and genes LMO4, PITX2a, TFAP2α, and Pax2 (Fig 8A, S7A and S7B Fig). Forced expression of PAX7-FOXO1 maintenance of a SOX2+ state (S7Ai,i',iii Fig), reduced the formation of HUC/D+ neurons (Fig 8 expression of the selected FP-RMS signature genes (Fig 8Aiv-iv",vi, S7Bi,i',iii,i iii',v-v" Fig). By contrast, PAX7-FOXO1 poorly induced LMO4 and PITX2 and TFAP2α (compare S7Bii,ii',iv,iv' to Fig 3Bii,ii',iv,iv'). This may stem from the differential transcriptional potential between PAX3-FOXO1 and PAX7-FOXO1.
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and a breakdown of the basal lamina (Fig 8Biv-iv'). The gain for PAX7 did not alter the distribution. Furthermore, PAX7-FOXO1+ cells gained the ability to colonize adjacent tissues, while the force of electroporated cells within the embryo (Fig 8Bv).

Finally, quantifying the number of PH3+ cells and the levels of phospho-RB1 in chick embryos expressing FOXO1 were consistent with these PAX variants reducing the ability of progenitors to proliferate (Fig 8Cii, S7C Fig); with the effects of PAX7-FOXO1 much stronger than that of PAX7. FOXO1+ cells was correlated with elevated levels of CDKN1c (Fig 8Ci, S7Dii,ii Fig). We next checked whether the proliferative behaviour of PAX7-FOXO1+ cells expressing CCND1 or MYCN (Fig 8Ci). At 24hpe in presence of either cell cycle regulator PAX7 rate as great as that of pCIG samples (Fig 8Ci).

Altogether these data demonstrate that the gross phenotypic traits provided by PAX3-FOXO1 are reminiscent, despite some differences in the molecular response of cells to the two factors. Ass underpinning these differences and how they impact the long term phenotype of cells could provide insight into the outcome between patients carrying either the t(2;13)(q35;q14) or t(1;13)(p36;q14) translocations.

Discussion

New markers of FP-RMS and their regulation by the PAX-FOXO1s

As for many cancers, the transcriptional state of FP-RMS varies considerably between patients, variations in the profile of expression of the embryonic muscle markers, MYOD1 and MYOG [64]. Transcriptome of 192 RMS patients we have been able to reevaluate the list of genes marking the distinctive feature of the FP-RMS molecular signature is its association with genes not only regulatory embryonic muscle cells, but also of other embryonic cell lineages, having in common a PAX3/7. Accordingly, the chromatin landscape of FP-RMS only partially matches that of myoblasts and requires PAX3-FOXO1 bound CRM in the vicinity of 40% of these FP-RMS associated genes and of PAX7/3; these CRMs represents a means by which PAX3/7 dependent developmental gene networks are expressed cells [11,12,70]. In addition, the presence of TFs known to also strongly influence the embryonic lineages [51,53–57,59,62,63,71] might also contribute to the FP-RMS dysfunctional.

Variations in the FP-RMS signature genes is likely to stem from the specific genetic aberrations genetic aberrations, and the cell of origin [5,6]. Our data demonstrate that the PAX-FOXO1s are tissue of some FP-RMS associated TFs, while their expression is normally silenced (Figs 3 and 4) and stem from a pioneer transcriptional activity [73], demonstrated for PAX3-FOXO1 in human. Recruitment of the fusion TF operates largely on closed and transcriptional shut down CRM. PAX3/7 TFs haven't yet been revealed. Yet, genetic studies suggest a model where cell fate specifically dependent transcriptional activation in the myogenic lineage (e.g. [75–77]) and in great means repression in the neural tube (e.g. [78–80]). As such PAX3 recruitment to the genome of myoblast marks [75] and PAX3 loss of function phenotypes in these cells can be largely rescued by FOXO1 [76]. Conversely, in the neural tube it would act at least partially as a transcriptional repressor to rescue Pax3 loss of function phenotypes [80]. Even more it can interfere with the normal function of key genes [29,80,81] and our study). In agreement, PAX3 poorly induced the activity of FP-RM...
embryos (Fig 4, S2B Fig). Interestingly, it has been observed that the fusion with FOXO1 inhibits therefore the PAX3-FOXO1 proteins harbour a higher transcriptional potential, which certainly non-specific co-factors.

The tissue specific activity of PAX3 can be bypassed, as demonstrated by the induction of MYC PAX3 is provided by RCAS based viral infection for 5 days[83]. This mode of transgenesis expends expression of the transgenes, as opposed to the electroporated and episomally trans progressively diluted by cell division. This is in line with the idea that the levels of PAX TFs are a response of cells. Exemplifying this, spinal progenitors harbouring different levels of PAX activity neuronal subtypes [84] and the loss of one single PAX3 allele leads to Waardenburg syndrome, [85].

The use of various promoters to drive the expression of PAX3-FOXO1 in zebrafish or the complete, expressed from one or two Pax3 alleles support the idea that PAX-FOXO1 levels are also instrumental in the transformation of healthy cells to FP-RMS like cells [28,29,32]. This may explain the discrepancies observed in the endogenous Pax3 locus or using Pax3 promoter region [81,84,86]. In these mouse models, specification of neuronal identities, the neural epithelium folding alterations and in some cases [86], reported in the presence of both myoblast and non myoblasts associated TFs expressing PAX3-FOXO1 and PAX7-FOXO1 support a routing of cells a FP-RMS like state.

Most importantly, our study, taken together with the study by Kendall et al. [29], supports the co-culture of FP-RMS from the cellular subtype from which FP-RMS can originate. Accordingly, 20–40% of primary tumour masses by NCC, such as the orbit, bladder, para-meningeal, head and neck areas ([41,46,87], S1A Fig) report the presence of FP-RMS primary growths in a giant naevus and spinal cord, that are uncommon [36,37]. This idea is further supported by the observation that the regulatory regions in the vicinity impacted by the t(2;13)(q35;q14) translocations remain active in the neural tube after the translocation origin on the manifestation of the disease and how much it can contribute the FP-RMS heterogeneity. In some cases, it is tempting to speculate that it will modulate tumour formation incidence, location and histology.

**PAX-FOXO1s mediated cell cycle inhibition limits the expansion of transformed and metastatic cells**

In the light of the cellular phenotypes appearing upon exposure to PAX-FOXO1, we propose the oncogenic drivers, whose activity is likely underpinning the timeline of tumour formation. On the other hand, our analyses revealed that the oncogenicity of PAX-FOXO1 transformed cell proliferation (Fig 5). Such negative effect of PAX-FOXO1s on cell cycle progression is unlikely to be a cell growth defect in the presence of the oncogenic drivers, namely EMT-TFs [89] (S1–S3 Tables, Fig 4).

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chromosome translocation is only seen in 30% of biopsies [5,6]. This calls for a better understanding of the cell cycle inhibition. The buffered cell cycle progression induced by PAX-FOXO1 could underlie the refractory response of FP-RMS cells to drugs such as CDK2 inhibitors [34] and also explain why tumours post-treatment [92], as shown for other cancers [2]. We propose that the decrease in the levels of the phospho-FOXO1 gain of function points at a decrease in the level of CDK2 activity and explains the arrested growth of FP-RMS cells to drugs such as CDK2 inhibitors [34]. Strong support for this hypothesis is also coming from studies that showed the resurgence of tumours post-treatment [92], as shown for other cancers [2].

Finally, amongst the approaches taken to study FP-RMS development and evolution [28–30,32] recapitulates the invasive and disseminative properties of PAX-FOXO1 expressing cells [92]. As human grafted cells [38], we believe that it will particularly suited for studying the modes of dissemination of FP-RMS transformed cells. Our model will also provide a means to investigate the molecular networks associated with PAX-FOXO1 mediated-latent metastatic state to overt metastasis [97]; and thereby to provide valuable insights into early therapeutic development.

**Methods**

**Bioinformatics**

Transcriptomes of FP-RMS and ERMS biopsies have been published elsewhere [42–46] (accession E-TABM-1202, E-MEXP-121 and data in [45]). Each dataset was based on Affymetrix microarray analysis (Sheet 2). Raw probe set signal intensities were normalized independently, using the free Bioconductor R package [98]. Individual expression matrices were merged and the residual technical variation was removed using the ComBat method implemented in the SVA R package [99]. Samples corresponding to the presence/absence of PAX3-FOXO1 or PAX7-FOXO1 fusion genes were subset from the original dataset. Differential analysis of fusion positive versus negative samples was conducted using the limma parameters: resp.type = "Two class unpaired", nperms = 100, random.seed = 37, testStatistic = delta score lower than 2.3 (FDR 0) where selected for subsequent analysis.

Hierarchical clustering of the normalized transcriptomes was implemented using the heatmap.2 function [101]. PAX3-FOXO1 chIPseq data (GSE19063, Cao 2010) were mapped to human genome (hg19) using the MACS2 [103] implemented on Galaxy server [104]. Peaks common to the 2 replicates in the RD cell line samples were selected using BEDtools [105] and annotated to the transcribed genes using the annotation tool of the PANTHER Classification System [107] or GSEA [108].

**Chick in ovo electroporation**

Electroporation constructs based on pCIG (pCAGGS-IRES-NLS-GFP) expression vector [109] of Pax3, Pax7, PAX3-FOXO1, PAX7-FOXO1 [80]; MYCN [68]; CCND1 [110]. Reporters for the human FOXO1 bound enhancers were cloned upstream of the thymidine kinase (tk) promoter and nuclelease promoter (mlp) and H2B-Turquoise. For detailed cloning strategies see supporting methods [109]. pCIG based constructs (1.5–2 μg/μl) were electroporated in Hamburger and Hamilton (HH) stages to describe protocols [112]. Embryos were dissected at the indicated stage in cold PBS 1X.
Embryos were fixed with 4% paraformaldehyde (PFA) for 45 min to 2 hr at 4°C, cryoprotected, embedded in gelatin, cryosectioned (14 μm), and processed for immunostaining [112] or in situ the reagents are provided in the S1 Methods. Immunofluorescence microscopy was carried out on microscope. Pictures of in situ hybridisation experiments were then taken with an Axio Observer microscope. Images were processed with Image J v.1.43g image analysis software (NIH) and Photoshop 7.0. All quantifications were performed using ImageJ v.1.43 g on usually more than 2 to 6 transverse sections per embryo. The number of cells positive for a marker per section was estimated on between 2 to 6 transverse sections per embryo. The number of sections taken into account the extent of electroporated cells found along the anterior-posterior axis of the embryo. The median is calculated and is represented with a dot on graphs. Fluorescence intensities in GFP+ cells were of interests whose size was adapted to that of cell nuclei and multi-measurement plug-in. These embryos and often in more than 5 embryos, the number of embryos analysed is always given in the intensities, the greatest variations in the data was set between cells and not between embryos. Developmental stage of each cells, their localisation within the neural tube and the levels of expression of similarity between two populations of values (i.e. between two types of chick sar Mann-Whitney U test in GraphPad Prism and all the p-values are given in figures legend. All quantifications were performed using as described previously [114] and with the Click-it EdU system (Thermo fisher).

EdU pulse labelling and staining

A solution of EdU 500uM was injected within the neural tube lumen 20h before harvest. Immunofluorescence microscopy was performed as described previously [114] and with the Click-it EdU system (Thermo fisher).

Cell dissociation from chick embryos

GFP positive neural tube regions were dissected after a DispaseI-DMEM/F-12 treatment (Stem Cell Technologies). Single cell suspensions were obtained by 3 minutes incubation in Trypsin-EDTA with mechanical pressure. Inhibition of Trypsin was ensured using with cold foetal bovine serum (FB S-100, Gibco). Dissociated cells were stained with 5uM Vybrant DyeCycle violet stain (V35003, Thermo Fisher). Staining was performed as described previously [99] in the dark. Light scattering parameters were quantified using a Cyan ADP flow cytometer were processed using Flowjo software v10.7.1 (Becton Dickinson, USA). Representative gating Single cell events were gated by forward scatter (FCS) peak vs Area (S6Ai Fig). Cells were also FSC Area vs Side Scatter (S6Aii Fig). FSC area vs GFP-log properties were used to segregate cells from GFP+ wild-type cells (S6Aiii Fig). Cell cycle analysis was performed by using the Deconvolution software with manual constraining G1 and G2 range for model fit optimisation (S6B Fig).
in each cell cycle phase was generated using Excel or GraphPad (Fig 6C; S6C Fig).

**GFP and DNA labelling and imaging 3D chick embryos**

Samples were incubated overnight with Atto488 (1/300, Sigma) at 4°C for GFP staining, washed 5–10 minutes in DRAQ5 (1/1000, Thermofisher) for DNA staining and finally washed in PBS. Samples were ventral side in 1% agarose for 3D imaging. 3D scans of samples were obtained with a 2-photon femtosecond pulsed Insight Spectra Physics laser, a Carl Zeiss 20x, NA 1.0 (water immersion BioTek) image acquisition software. A single wavelength of 930nm was used for exciting all fluorophores. Two GaAsp sensitive photomultipliers allowed simultaneous detection of the two emission lights through a dichroic mirror 585nm and a bandpass filter 525/50nm.

**3D images processing and quantitative analyses**

Image pre-processing and segmentation were performed using ImageJ and Imaris. Background channel to eliminate autofluorescence coming from the tissue. Bleach correction normalizing the thickness was performed on DRAQ5 channel stacks of thick samples, notably 72hpe samples. An automatic surface segmentation plugin based on intensity and size (<95 voxels) allowed removal of dead cells x,y,z coordinates of the centre point, the major axis of their ellipsoid fit, the sphere radius retrieved for all segmented nuclei. The surfaces encompassing the neural tube, the neural cavity were delineated on the DRAQ5 signal, on x-y planes every 3 z-stacks. Distance Transformation segmentation was used to quantify the distance between the centre of the nuclei and this cavity. Cell orientation was established by converting vector representing the major axis of the ellipsoid fit of GFP positive cells into polar coordinates.

**Imaging the apical surface of Par3 and GFP labelled spinal cord**

Dissected spinal cords were fixed in PFA4% for 1h and washed in PBS. Immunofluorescences were performed on the samples flatten between a slide and coverslip was imaged using (Leica DMi8: CSU-W1 Yokogawa spinning disk) and MetaMorph (Molecular Devices) image acquisition software.

**Supporting information**

**S1 Fig.**
(A) Body locations of RMS biopsies. Locations of FP-RMS (red) and FN-RMS (blue) biopsies assessed in Fig 2A and coming from previous studies [42–46]. ND: Non determined. (B) PITX2 RMS from FN-RMS cells. (i, iii) Pictures of western blots using the indicated antibodies on pro FN-RMS (RD, RDAbl, Rh36) and FP-RMS (Rh3, Rh5, SJRH30, Rh4) cell lines and (ii) normaliz GAPDH. This shows variable levels of PAX3-FOXO1 (i, ii) between FP-RMS cell lines and of MEFs. In addition, specific ectopic expression of several PITX2 isoforms (iii) in FP-RMS versus FN-RM to S4 Raw images). [https://doi.org/10.1371/journal.pgen.1009164.s001](https://doi.org/10.1371/journal.pgen.1009164.s001)

**S2 Fig.**
(A) (i-ii') Immunodetection of GFP and PAX2 on transverse sections of chick embryos 48hpe with high magnification. (A) (i-ii') Immunodetection of GFP and PAX2 on transverse sections of chick embryos 48hpe with high magnification.

Quantification of PAX2 expression levels in GFP+ cells in the spinal cords of chick embryos 48hpe with high magnification. (B) Immunodetection of GFP and PAX2 on transverse sections of chick embryos 48hpe with high magnification.
chick embryos 48hpe with the indicated plasmids. x” panels are views on the myotome (myo) of Immunostaining for GFP, Turquoise direct fluorescence and DAPI staining on transverse sections indicated plasmids and a reporter for human PRDM12CRM and CDH3CRM. (Civ) Quantification that of GFP in cells electroporated with PRDM12CRM reporter at 24hpe (dots: single cell values embryos). Mann-Whitney U test p-value: ****: p< 0.0001. Scale bars: 50µm. https://doi.org/10.1371/journal.pgen.1009164.s002 (TIF)

S3 Fig. Cell shape and orientation dynamics induced by PAX3-FOXO1.
(A) (i-iii) Projection of 3D images of embryos 48hpe with the indicated plasmids, stained with D (i’-iii’i) Result of the segmentation performed at the level of the boxes indicated on samples i-iii. electroporated half of the neural tube are transparent yellow, while cell nuclei are coloured. In p segregating progenitor nuclei from neurons is highlighted in transparent red. (iv) x (medial-later (dorsal-ventral) axes giving the orientation of i-iii samples. (B) (i-iii) Representative 3D shape of scanned whole embryos 48hpe with the indicated plasmids. (iv) Temporal dynamics of the ellip segmentation of GFP+ nuclei (as shown in i-iii) in whole-mount embryos (mean ± s.d., n>6 embryos) dimensions of the chick embryos of θ and φ polar angles of the vector (blue arrow) defining the circle). (ii-iii) φ (ii) and θ (iii) possible values and major axes of chick embryos (black circles) an electroporated with the indicated plasmids at 48hpe. https://doi.org/10.1371/journal.pgen.1009164.s003 (TIF)

S4 Fig. Extended characterization of the epithelial-mesenchymal transition triggered by PAX3-FOXO1.
(A) Immunodetection of GFP, PARD3, activated βCATENIN (βCAT.) and β1-INTEGRIN on trans 48hpe with the indicated plasmids. In i and ii, x’ and x’’ panels are blown up on a subset of x panel GFP+ are blown up on a subset of x panel GFP+ and GFP− cells. Arrowheads in x’ panels point are on the basal side of cells. Arrows in iii’ indicate increased levels of β1-INTEGRIN at the membrane Scale bars: 50µm. (B) Normalized levels of SNAI1 and ADAM10 mRNA assayed by DNA microbiopsies (dots: RNA sample values; bars: mean ± s.e.m.; Mann-Whitney U test p-value: ****: p< https://doi.org/10.1371/journal.pgen.1009164.s004 (TIF)

S5 Fig. Cell cycle state of PAX3 and PAX3-FOXO1 overexpressing embryonic spinal cells.
(A) (i-iii*) GFP and activated CASPASE3 immunodetection and DAPI staining on transverse sections the indicated plasmids. (iv) Quantification of the number of activated CASPASE3+ cells in the C population in embryos 24hpe with the indicated plasmids (dots: embryo values; bar plots: mean ± s.e.m.). (B) GFP and MCM staining on transverse sections of chick embryos 48hpe with the indicated plasmids. (iv) Quantification of the number of cells 48hpe with the indicated plasmids (dots: single cell values; bar plots: mean ± s.e.m.; n>5 fold changes in the expression of the indicated genes relative to their mean expression in pCIG GFP+ from chick embryos 48hpe with the indicated constructs. (D) Quantification of the number of cells 48hpe with the indicated plasmids (dots: embryo values; bar plots: mean ± s.e.m.; n>5 fold changes in the expression of the indicated genes relative to their mean expression in pCIG GFP+ from chick embryos 48hpe with the indicated constructs. Mann-Whitney U test p-value: *: p< 0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001. Scale bars: 50µm. https://doi.org/10.1371/journal.pgen.1009164.s005 (TIF)

S6 Fig. Cell cycle phases of PAX3 and PAX3-FOXO1 overexpressing cells.
(A) FACS gating strategy in 3 steps using Flowjo: (i) isolation of singlets (FS: forward scatter/ap (ii) selection of cells based on their size (FS: forward scatter) and granularity (SS: side scatter);
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