Haploinsufficiency of the lysosomal sialidase NEU1 results in a model of pleomorphic rhabdomyosarcoma in mice

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Rhabdomyosarcoma, the most common pediatric sarcoma, has no effective treatment for the pleomorphic subtype. Still, what triggers transformation into this aggressive phenotype remains poorly understood. Here we used Ptch1+/−/ETV7TG/+− mice with enhanced incidence of rhabdomyosarcoma to generate a model of pleomorphic rhabdomyosarcoma driven by haploinsufficiency of the lysosomal sialidase neuraminidase 1. These tumors share mostly features of embryonal and some of alveolar rhabdomyosarcoma. Mechanistically, we show that the transforming pathway is increased lysosomal exocytosis downstream of reduced neuraminidase 1, exemplified by the redistribution of the lysosomal associated membrane protein 1 at the plasma membrane of tumor and stromal cells. Here we exploit this unique feature for single cell analysis and define heterogeneous populations of exocytic, only partially differentiated cells that force tumors to pleomorphism and promote a fibrotic microenvironment. These data together with the identification of an adipogenic signature shared by human rhabdomyosarcoma, and likely fueling the tumor’s metabolism, make this model of pleomorphic rhabdomyosarcoma ideal for diagnostic and therapeutic studies.
Genetic, epigenetic, and environmental cues shape molecularly distinct subpopulations of cells in an evolving solid tumor. This intratumoral heterogeneity is a likely prelude to chemotherapy resistance and metastases. Rhabdomyosarcoma (RMS) is the most common pediatric soft tissue sarcoma with poor outcomes for high-risk patients. Although it usually displays myogenic features, RMS can originate from both myogenic and non-myogenic progenitor cells, which explains, at least in part, its different sites of occurrence (e.g., limbs, chest, head and neck, and retroperitoneum). Generally, these tumors are classified into two major histological subtypes: (1) ERMS (embryonal RMS) (~60%) has a mostly favorable outcome, which decreases to only 40% overall survival when metastatic; (2) ARMS (alveolar RMS) (~20%) is the aggressive form associated with early metastatic dissemination and poor prognosis. More than 80% of ARMS are defined by two chromosome translocations, involving PAX3/7 and FOXO1. Both ERMS and the rare pleomorphic RMS subtypes lack these chromosome translocations and develop into genetically more complex tumors, frequently showing inactivation of the p53 pathway and/or another oncogene driver mutations. These tumors express both developmentally early myogenic markers (e.g., PAX3/7, MyoD, and Myogenin) and markers of terminal differentiation (e.g., Desmin and SMA). Although several mouse models of ERMS have been reported, none addressed the molecular and cellular events that drive RMS into a pleomorphic state, which therefore remains poorly understood.

We have shown earlier that haploinsufficiency of the gene encoding the lysosomal sialidase Neu1 in Arf−/− mice rendered different types of sarcomas more aggressive. Neu1 hydrolyzes terminal sialic acids from sialylated glycoproteins, changing their biochemical properties and affecting downstream pathways. One of these pathways is lysosomal exocytosis which Neu1 negatively regulates by cleaving the sialic acids of LAMP1. This lysosomal membrane protein is responsible for docking lysosomes at the plasma membrane (PM) of cells, prior to their fusion with the PM. Low Neu1 activity increases the number of lysosomes, decorated with a long-lived, sialylated LAMP1, that dock at the PM. This ultimately results in excessive lysosomal exocytosis with deleterious consequences for the integrity of PMs and the extracellular matrix (ECM). The common readouts of this aberrant process in cells are increased activity of lysosomal enzymes (e.g., β-hexosaminidase) extracellularly and relocation of LAMP1 to the PM.

Dysregulated lysosomal exocytosis is the basis of disease pathogenesis in Neu−−/− mice, a model of the lysosomal storage disease sialidosis. Relevant to these studies is the muscle connective tissue phenotype in Neu−−/− mice that undergo a precancerous transformation, leading to expansion of the tissue via increased proliferation and generalized fibrosis. Neu1 deficient fibroblasts behave as myofibroblasts/mesenchymal cells, being simultaneously proliferative and migratory/invasive. They show increased exocytosis of soluble proteolytic enzymes and exosomes, which together remodel the ECM and propagate the fibrotic disease. We found that this pathogenic cascade accelerated the occurrence of several types of sarcomas in Neu1+/−/Arf−/− mice and transformed these tumors into an aggressive and chemoresistant phenotype. We sought to explore these Neu1-mediated phenomena in a mouse model of RMS.

The Patched1 (Ptc1)+/− mice, a model of medulloblastoma, also develops ERMS-like tumors at low incidence. These tumors are driven by activation of the Shh pathway, which is normally kept inactive by binding of the Ptc1 receptor to Smo (smoothened). Reduced Ptc1 expression frees Smo, which then hyperactivates Gli transcription factors, triggering growth and proliferation. Crossing the Ptc1+/− mice with a transgenic mouse line (ETV7TG+/−) expressing human ETV7 increased the incidence and penetrance of ERMS and hematopoietic malignancy. ETV7, an ETS transcription factor, promotes the assembly of a novel rapamyacin-resistant mTORC3 complex, by directly binding to mTOR. ETV7 is differentially expressed in all human RMS, and mTORC3 increases the proliferation of several human RMS cell lines. Furthermore, it was shown that the higher incidence of RMS in Ptc1+/−/ETV7TG+/− mice is driven by mTORC3.

Here, we show that reducing Neu1 expression in the Ptc1+/−/ETV7TG+/− mouse model changes RMS tumors into a pleomorphic state. These tumors have increased cell heterogeneity, a distinctly fibrotic microenvironment, and share features with both human ERMS and ARMS. We found that deregulated lysosomal exocytosis downstream of low Neu1 is the underlying pathway driving transformation in these tumors, which are also fueled by an adiogenic signature. Thus, Neu1+/−/Ptc1+/−/ETV7TG+/− mice are a model of human pleomorphic RMS and may represent a powerful means for diagnostic and therapeutic studies of these aggressive and incurable tumors.

Results

Neu1 haploinsufficiency promotes the development of poorly differentiated RMS in the Ptc1+/−/ETV7TG+/− mouse model. In Neu1+/−/Ptc1+/−/ETV7TG+/− (NPE) mice, low Neu1 RNA and protein expression (Supplementary Fig. 1a, b) was sufficient to increase the incidence of RMS to 62%, as compared to 54% in the Ptc1+/−/ETV7TG+/− (PE) mice and only 8% in the Ptc1+/−/− mice in 2 years (Fig. 1a). RMS was diagnosed based on morphology and immune reactivity to the myogenic regulatory factors (Mrfs) MyoD and Myogenin, and the muscle-specific type III intermediate filament desmin (Des) (Supplementary Fig. 1c–e). The survival rate of both NPE and PE mice was comparable but significantly lower than that of Ptc1+/−+/− mice (Fig. 1b). All RMS tumors in NPE and PE models developed in either the extremities or the trunk, reflecting the location of human ARMS and pleomorphic RMS with poor prognosis and short survival.

Within the cohort of mice with RMS tumors 4/35 (11.4%) NPE mice and 8% in the PE Model changes RMS tumors into a pleomorphic state. These tumors are synchronous rather than metachronous. We also found that ETV7 expression in both NPE and PE tumors, as compared to the NPE model changes RMS tumors into a pleomorphic state.
An algorithm designed to quantify nuclear morphometry in tumor H&E sections revealed an increased number of aberrantly large, multinucleated cells with atypical mitotic figures, predominantly in NPE tumors (Fig. 1i). Because of the very large size of most of the nuclei in the NPE tumors, their total number per annotated area was lower than that in PE tumors (Fig. 1j). Instead, other typical RMS features were common to both NPE and PE tumors, including spindle-shaped cells, round rhabdomyoblasts, cross-striated mature skeletal myocytes and myofibers (Fig. 1g, h), as well as expression of MyoD, Myogenin, and Desmin (Supplementary Fig. 1e)\(^3\). Together, these results suggest that Neu1 haploinsufficiency superimposed on the spontaneously occurring PE tumors changes the overall morphology and differentiation status of RMS.
Neu1-mediated pleomorphism is associated with neoplastic fibrosis. In general, aggressive/invasive tumors undergo active remodeling of their ECM, which creates a fibroctic microenvironment that facilitates metastatic spread and promotes drug resistance. To assess the extent of collagen deposition and ECM remodeling, Masson’s trichrome staining identified extensive areas of collagenous connective tissue that were much more prominent in the NPE (blue) than in PE tumors, particularly at invasive fronts and pleomorphic foci (Fig. 2a, b). A similar increase in connective tissue was observed in Masson’s trichrome-stained human tissue microarrays (TMAs) from several RMS patients (ERMS n = 16, ARMS n = 17, and spindle cell/sclerosing RMS n = 3), where 50% of all TMA cores had more than 9% of the total area as collagenous material, with an average of 28.7% in ERMS, 9.7% in ARMS, and 23.3% in spindle cell/sclerosing RMS, although the latter only comprised three samples (Fig. 2c and Supplementary Fig. 3a). In addition, we observed a substantial number of cells that showed intracytoplasmic collagen as well, with an average of 12.8% in ERMS, 8.5% in ARMS, and 13.7% in spindle cell/sclerosing RMS (Supplementary Fig. 3b). Quantification of the staining of the mouse tumors revealed a significant increase of connective tissue in the NPE tumors compared to PE tumors, underscoring their more aggressive nature (Fig. 2d).

Similar to what is seen in human RMS tumors, subpopulations of NPE cells also showed quantifiable intracytoplasmic collagen (Fig. 2e and Supplementary Fig. 3b), indicating their cell-autonomous capacity to deposit ECM, like that of myofibroblasts during fibrosis. The latter was further confirmed by the significant upregulation of Col1a2 and Col4a1 expression in NPE versus PE RMS (Fig. 2f, g). These combined observations were not seen in normal muscle tissue of these mice (Supplementary Fig. 3c–e), emphasizing that the connective tissue deposition observed in the NPE RMS tumors was caused by the combination of reduced activity of Neu1 within tumor cells and stroma. Thus, reduced Neu1 expression promotes pleomorphism and transforms the tumor stroma into a desmoplastic, fibrotic state.

To assess the molecular events that might contribute to the aberrant histopathology and fibrotic state of NPE tumors, we focused on exacerbated lysosomal exocytosis. Testable readouts of this dysregulated process are an accumulation of a long-lived, sialylated LAMP1 due to low Neu1 activity, and LAMP1 redistribution at the cell surface. We exploited both these parameters to measure the expression levels of LAMP1 as well as the fraction of this protein at the cell surface in human TMAs from several RMS patients. Matched immunostained tissue cores from RMS patients showed that very low expression of Neu1 was accompanied by an overall increase in LAMP1 (Fig. 3a, Supplementary Fig. 3a). Using a membrane immunoreactivity computer learning algorithm applied to TMA cores, we quantified LAMP1 positive staining at the cell membrane (LAMP1PM) in tumor sections of NPE and PE mice. Mean ± s.d.; Student’s (unpaired) t-test; PE: n = 10 and NPE: n = 6 biologically independent tumors.
activity of β-hexosaminidase was measured in the interstitial fluid of several NPE RMS compared with PE samples (Fig. 3h), confirming that NPE tumors were more exocytic.

Single-cell multiplex flow cytometry underscores the complex cell heterogeneity in Neu1<sup>+</sup>/Ptc1<sup>+</sup>/ETV7<sup>TG−</sup> tumors. Based on the combined features of increased Lamp1<sup>PMpos</sup>, lysosomal exocytosis, and pleomorphism in limb and trunk NPE compared with PE RMS, we designed a multiplex high parameter flow cytometry strategy that evaluated Lamp1<sup>PMpos</sup> cells separately from Lamp1<sup>PMneg</sup> cells within three main cell populations: cancer, stroma, and hematopoietic cells (Fig. 4a). Protein expression data were generated by using Lamp1<sup>PM</sup> in combination with markers selective for each cell population<sup>35,38–50</sup>.

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followed by tSNE analysis. The distribution and density of cells in limb and trunk RMS from NPE and PE mice highlighted the extent of cell heterogeneity (Fig. 4b). To uncover distinct cell populations within each tumor sample, we narrowed the tSNE analysis to tumor and stromal cells by excluding hematopoietic cells, using CD45 (Supplementary Fig. 6). Several discrete cell clusters were identified, which differed not only between NPE and PE RMS, independently of their CD45 status, but also between limb and trunk tumors (Fig. 4c). This may be linked to the cell of origin that supports the development of tumor versus limb tumors, but a more comprehensive investigation would be necessary to confirm these differences.

**Lamp1 at the plasma membrane marks highly exocytic and undifferentiated cell populations in Neu1+/−/Ptch1+/−/ETV2TGF−/− tumors.** tSNE analyses of the selected CD45neg cell populations in limb and trunk tumors of both NPE and PE mice (Fig. 4b, c) again revealed distinct cell density and distribution (Fig. 5a, b). These cell populations were further analyzed by using Lamp1PM expression in combination with a set of canonical markers distinct for cancer and stromal cells (i.e., desmin, Ki67, Sca1, Sma, Pcam1, CD44, Lyve1, and EpCam) (Fig. 5c–h). This allowed for the annotation of 26 and 22 different cell clusters in limb and trunk tumors, respectively (numbered in Fig. 5c–h). Within the annotated populations, the total percentage of Lamp1PMpos exocytic cells was 45.95% in limb (cell clusters 1–12) and 11.14% in trunk (cell clusters 1–10) RMS (Fig. 5e–h). In both limb and trunk RMS a clearly larger percentage of Lamp1PMpos cells was detected in NPE (cluster 1–12) than in PE (cell clusters 1–10) tumors (Fig. 5e, f). Most importantly, the majority of Lamp1PMpos cells showed pleomorphic features (cell clusters 1–6) and expressed simultaneously the terminal (myo)differentiation marker desmin and markers of progenitor or undifferentiated cells, such as Ki67 (proliferation), Sca1 (progenitor), Sma (smooth muscle actin/mesenchymal), Pcam1 and CD44 (endothelial), and Lyve1 (lymphatic endothelial) (Fig. 5c–f). These exocytic, pleomorphic subpopulations accounted for 21% and 15% of cancer cells in the limb and trunk (cell clusters 1–6), respectively, and were more abundant in NPE than PE RMS (Fig. 5e–h). The combination of pleomorphic and exocytic cells enriched in NPE tumors indicates that Neu1 haploinsufficiency promotes the poorly differentiated state of the tumors, albeit an in-depth explanation of this phenomenon may require further investigation.

Similarly, within the stromal CD45neg and Desneg cell populations, the majority of Lamp1PMpos cells included cancer-associated fibroblasts (SMA) (cell cluster 7–9) and epithelial cells (cell clusters 10–11 in limb and 10 in trunk), but also expressed markers typical of myofibroblasts/mesenchymal cells, e.g., Ki67, Sca1, Sma, Pcam1, CD44, Lyve1, and EpCam (epithelial) (Fig. 5e–h). Again, these proliferative, exocytic stromal cells were mostly enriched in NPE limb (cell cluster 7) and trunk RMS (Fig. 5e–h). Curiously, a small percentage of endothelial cells with progenitor features (Pcam1pos, Sca1pos) was almost exclusively present in NPE tumors (cell cluster 22 in limb and 15 in trunk) (Fig. 5e–h). Expression levels of markers defining tumor and stromal cells were also assessed by tSNE heatmaps that demonstrated the higher signal intensity of some of the markers (Des, Sma, Sca, Pcam1, Lamp1, and Ki67), specifically in NPE RMS (Supplementary Fig. 7a, b). These tumors also contained a higher percentage of CD45pos macrophages, T cells, and B cells, than PE tumors (Supplementary Fig. 7c–f), likely contributing to an inflammatory pro-tumorigenic microenvironment. However, a more detailed analysis of the immune infiltration would be required to tease out its function in these tumor models. These results, based on single-cell protein expression, suggest that in NPE tumors, not only cancer cells but also stromal cells are maintained in an intermediate state of transdifferentiation that could be the basis of their distinct pleomorphic phenotype.

**Pleomorphic Neu1+/−/Ptch1+/−/ETV2TGF−/− RMS develop an adipocytic metabolic signature.** Although the cell composition of NPE and PE limb and trunk tumors was similar, there was a distinctly higher percentage of cell populations expressing specific combinations of markers in NPE vs PE tumors (Fig. 5e, f). Taking the latter into account, we queried microarray data from individual NPE and PE limb and trunk RMS in search of distinct gene expression signatures that would add to their respective protein expression profiles and support pleiomorphism. The microarray analysis allowed for the detailed identification of differentially expressed genes between NPE and PE tumors. The volcano plot and heatmap (Fig. 6a, Supplementary Fig. 8a) comprising a cohort of limb and trunk RMS showed that, compared with PE, NPE tumors had 491 upregulated and 287 downregulated genes (Fig. 6a, Supplementary Fig. 8a, Supplementary Data 3). Using Enrichr,21,32 we found that most of the upregulated genes in NPE RMS belong to pathways of lipid metabolism, myogenesis, and epithelial-to-mesenchymal transition (EMT) (Supplementary Fig. 8b, Supplementary Data 4), whereas genes downregulated in NPE belong to the following pathways: matrix metalloproteinases, cytokine-cytokine receptor interaction, HIF-1 signaling pathway and lung fibrosis (Supplementary Fig. 8c, Supplementary Data 5). GSEA (geneset enrichment analysis) of the total genetic landscape of limb and trunk RMS from NPE and PE mice identified the following geneset libraries: the KEGG PPAR (peroxisome proliferator-activated receptor), the Gene Ontology (GO) fatty acid metabolic process, the GO brown fat cell differentiation, and the KEGG vascular smooth muscle contraction (Fig. 6b). The leading edge of all four libraries/pathways positively correlated with NPE upregulated genes (Fig. 6b). White, beige and brown adipogenesis, and fatty acid metabolism are regulated by the aforementioned GSEA pathways, Pparγ was one of the most upregulated genes in NPE RMS (Fig. 6b, Supplementary Data 6), together with some of its direct target genes, e.g., Fabp4, (fatty-acid-binding protein 4 or adipocyte protein 2), Cd36 (cluster of differentiation 36), AdipoQ (adiponectin), and its receptor AdipoR1, but not AdipoR2 (Fig. 6a, c, Supplementary Data 6).
The derivation of brown adipocytes from skeletal muscle precursors or beige adipocytes from white adipocytes is orchestrated by PPARγ recruitment of PRDM16 (PR (PRD1-BF1_RIZ1 homologous)-domain-containing 16), which together form a core transcriptional complex. PPARγ also recruits EBF2 (the early B-cell factor 2), a selective determinant of brown and beige adipocyte precursors, and coactivates the expression of UCP1 (uncoupling protein 1 of brown adipocytes), PPARA, and PRDM16. We found that in NPE RMS, Pparg, Prdm16, and Ebf2 were upregulated together with their target, Ucp1 (Fig. 6c, Supplementary Data 6). It is well documented that beige adipocytes that transdifferentiate from white adipocytes are derived from mesodermal stem cells expressing Pdgfra, Pdgfrb, Acta2, and Myh11, whereas brown adipocytes share with skeletal muscle cells the combined markers of dermomyotome precursors, Myf5 and Pax7. In our NPE RMS, only Acta2 and Myh11 were
significantly upregulated but not Pdgfra, Pdgfrb, Myf5, or Pax7 (Fig. 6d, Supplementary Data 6), suggesting a preferential presence of white and beige adipocytes.

The earliest progenitor marker of myogenesis, Pax3 was downregulated in NPE vs PE RMS, while Pax7 and Myf5 showed no significant difference in expression (Fig. 6d). The other upstream regulators of myogenesis, MyoD and Myog, were significantly increased in NPE RMS (Fig. 6d), while the terminal differentiation gene Des was downregulated (Fig. 6d). Additionally, Myocd and Myh11, both smooth muscle specification genes, were highly upregulated in the NPE compared to PE RMS (Fig. 6d, Supplementary Data 6); Myh11 was also found in the leading edge of the KEGG vascular smooth muscle contraction pathway (Fig. 6b, Supplementary Data 6). Notably, human MYH11 protein, which is normally not expressed in RMS, is a marker of sarcoma pleomorphism, and an effector of lysosomal exocytosis downstream of Neu1 downregulation. So far, these results suggest that NPE RMS are already committed to a myogenic fate without reaching full differentiation. Moreover, the upregulation Myh11 reinforces the notion that these tumors are both pleomorphic and exocyctic.

Although NPE and PE RMS depend on the combination of Ptch1<sup>−/−</sup> and ETV7G<sup>+/−</sup> for tumor specification and growth, the addition of Neu1 haploinsufficiency drastically increased Myc.
**Fig. 5** Lamp1PMpos cells are enriched in exocytic undifferentiated NPE RMS. **a, b** tSNE graphs showing density and distribution of CD45neg cells from limb (a) and trunk (b) RMS of PE (blue) and NPE (red) mice. **c, d** tSNE of single-cell analysis of limb (c) and trunk (d) representation of the distinct cell clusters identified in RMS (numbered and colored) with the markers used with CD45neg cells from PE and NPE mice. **e, f** Annotated limb (e) and trunk (f) RMS CD45neg exocytic Lamp1PMpos and Lamp1PMneg cell populations from PE and NPE mice. Bubble map indicates the percentages of cells from PE and NPE contributing to a cell cluster. Heatmap shows the signal intensity of expression of the different markers used. **g, h** Pie graph of CD45neg cells from limb (g) and trunk (h) PE and NPE RMS showing the percentage of each cell cluster (numbered and colored matched).
expression, the marker of proliferation, and the downstream effectors of the SHH pathway, Gli1 and Gli2, known to be associated with metastatic growth (Fig. 6e). In agreement with the status of partial differentiation and smooth muscle/mesenchymal characteristics of NPE RMS, genes involved in epithelial-to-mesenchymal transition (EMT) and metastasis i.e., Snai1, Snai2, Vcam1, Upk1b, and Prss35, were also significantly upregulated (Fig. 6e). It is noteworthy that the top-upregulated gene in NPE vs PE RMS is H2Eb1, encoding the mouse histocompatibility class II antigen E beta (MCH II), which is involved in antigen presentation, CD4+ T cell recognition and activation of the immune response60 (Fig. 6a). The relevance of this observation will require extensive future study exploring the immune response component of these tumors, which is beyond the scope of this work.
To exclude the possibility that *Neu1* haploinsufficiency by itself can cause the significant changes in gene expression observed between PE and NPE tumors, we performed RT-qPCR analysis of the same set of genes in normal muscle tissue from both NPE and PE mice and found no differences (Supplementary Fig. 9a–c). Overall, our results indicate that NPE RMS maintains a status of intermediate differentiation and pleomorphism fueled by a strong adipogenic component, likely linked to *Neu1* haploinsufficiency.

**Discussion**

Pleomorphic RMS is a rare and aggressive form of sarcoma, occurring mostly in adults that is often difficult to diagnose and treat. These tumors present with a mixture of large, atypical pleomorphic rhabdomyoblasts, often multinucleated, and partially differentiated spindle-like cells.

By lowering the expression levels of the lysosomal sialidase *Neu1* in the *Ptx1*+/−/*Etv7*+/− genetic background, we have successfully developed a spontaneously occurring mouse model of pleomorphic RMS without the use of conditional alleles. It is important to emphasize that the effect of *Neu1*+/− is not in the generation of RMS, but rather in its pleomorphic transformation once the tumor is initiated; while *Etv7* expression is promoting a high incidence of RMS formation in *Ptx1*+/− mice. Although NPE tumors arise preferentially in the limbs and trunk and only express 10% of the genes that trended with human RMS, their genetic features are much closer to ERMS. Unfortunately, the unavailability of compatible genetic data from human pleomorphic RMS that we could compare to our NPE datasets, limits our current evaluation of NPE tumors to their histological characteristics, and further investigation is required to confirm NPE mice as a model of human pleomorphic RMS.

Exploiting the low NEU1 > high lysosomal exocytosis axis, we developed a single-cell approach that relied on the increased levels of the Neu1 substrate Lamp1 at the PM of cancer and stromal cells. This approach allowed us to pinpoint tumor heterogeneity, consisting of specific cell populations that are highly exocytic and likely responsible for initiating/perpetuating RMS transformation and ECM remodeling. The ensuing fibrotic microenvironment surrounding pleomorphic foci in NPE RMS is typical of the most aggressive and chemo-resistant tumors in humans.

The presence of mature adipocytes together with adipose-like cells in both murine and human RMS could be explained by a shared developmental ancestry between skeletal muscle and brown fat. However, brown adipocytes originate from early dermomyotomes (Pax3+/En1+/Myf5+), while distinct somite populations expressing *MyoD* downstream of *Myf5* are already committed to myogenesis. Therefore, fate determination between brown adipocytes and committed somite populations should happen prior to *MyoD* expression. Given the high levels of *MyoD* detected in NPE RMS, we can infer that these tumors have reduced brown adipocytes. In contrast, they might be enriched in beige adipocytes, since these derive from progenitors expressing Sma, Myh11, Pdgfra, or Pdgfrb, and transdifferentiate from mature white adipocytes stimulated, among others, by PPARγ activation and release of adipokines, including adiponectin.

In this scenario, tumor cells, cancer-associated fibroblasts, and cancer-associated adipocytes may together sustain cancer progression and chemo-resistance by fueling metabolism and maintaining a pro-fibrotic microenvironment downstream of low NEU1 activity. These combined features, largely depending on posttranslational modifications of glycans, appear to be common between our NPE model and human RMS, given that increased
adipocytes have been detected in poorly differentiated, human metastatic or recurrent RMS, as well as in chemotherapy-treated RMS\textsuperscript{66–68}. We propose that the NPE mice can be exploited as a preclinical model of pleomorphic RMS to understand the pathology of these aggressive tumors and explore new therapeutic opportunities in pathways controlled by NEU1. Furthermore, the overlooked adipose component in these tumors underscores the importance of combining the use of adipose markers during the diagnosis of this type of RMS.

**Methods**

**Mice.** All procedures were performed following NIH guidelines and animal protocols approved by the St Jude Children’s Research Hospital Institutional Animal
Fig. 7 Adipogenic features of NPE RMS are shared with human RMS. a Representative micrographs of increased adiponectin immunostaining in NPE versus PE RMS. Increased cell surface positive staining is depicted in the zoom inset of NPE RMS. Scale bar: 25 μm. b Representative micrograph of strong adiponectin immunostaining in a core of an undifferentiated human RMS from 2 TMAs; n = 40 biologically independent samples. Intense staining is depicted in the zoom inset. Scale bar: 25 μm. c, g Representation of genetic expression of ADIPOQ (c), PPARG (d), CD36 (e), MYH11 (f), and UCP1 (g) across human patient RMS RNAseq data (PCGP). Gene expression is represented in relation to the mean value. All samples of ERMS (blue), ARMS (orange) and undefined RMS (black) are analyzed shown first on the x-axis (RMS), followed by the representation of the cluster of the top five samples with the highest ADIPOQ expression (Top 5). n = 44 biologically independent samples. h, i Comparison of ADIPOQ (h), PPARG (i), CD36 (j), MYH11 (k), and UCP1 (l) expression between ERMS and ARMS patients. Mean ± s.d; Mann–Whitney U-test used to compare ERMS and ARMS; n = 27 and n = 14 biologically independent samples, respectively.

Care and Use Committee (IACUC); animal protocol #235–100636. Neu1+/−/FVB/NJ mice were crossed with Pithc+/−/ETV7TG/− (/129sv/C57BL/6) mice32. The mice, both males, and females (1 month–24 months), used in this study were obtained after 8 timespan (2 years) from the same strain breeding schedule: Neu1+/−/Pithc+/−/ETV7TG/− X Neu1+/−/Pithc+/−/ETV7TG/−, but PE and NPE mice were not necessarily came from the same litter. Mice presenting with masses were observed for lethargy and sacrificed when moribund, or if they displayed symptoms such as paralysis or when the tumor volume reached 20% of body mass in accordance with IACUC-approved guidelines.

Real-time quantitative polymerase chain reaction. Total RNA was isolated from mouse tumors using the PureLink RNA Kit (Life Technologies), and DNA contaminants were removed on a dextran sulfate 1 column (Life Technologies), according to the manufacturer’s protocol. RNA quantity and purity were measured using a NanoDrop–Lite spectrophotometer (Thermo Fisher Scientific). Complementary DNA was synthesized using 0.5–5 μg of total RNA with RT2 First Strand Kit (Qiagen). RT–qPCR was performed using RT2 SYBR Green Mastermix, 1 μl of cDNA, 10 μM primers, and 1× RT2 Mastermix in a total volume of 20 μl. Fold decrements were calculated by comparing the level of fragmental expression after normalization to the level of expression of CD271, which was used as an endogenous control. All reactions were run in triplicate on the real-time PCR machine (Bio-Rad). Samples were normalized to 18S ribosomal RNA. The specific primers used are summarized in Supplementary Table 1.

Western blotting and co-immunoprecipitation. Frozen tumors were powdered by grinding in liquid nitrogen and homogenized in 1× Cell Signaling lysis buffer [20 mM tris–HCl, pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM β-glycerophosphate, 1 mM Na3VO4, and leupeptin (1 μg/ml) (Cell Signaling Technologies)] supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), for 2 min at 30 °C in the Omni Prep Multi-Stack homogenizer (QIAGEN). Lysates were spun through a QIAshredder column (QIAGEN), and freeze-thawed three times. After centrifugation at 20,000 × g for 30 min at 4 °C, the protein concentration in the supernatant was determined using a BCA protein assay kit (Bio-Rad). Per sample, 750 μg of protein was subjected to 4%–12% bis-tris protein gels (Life Technologies) at 12% SDS–gel (Life Technologies). Proteins were transferred onto nitrocellulose membranes using the iBlot system (Life Technologies) following the manufacturer’s protocol. Membranes were blocked with 5% milk and 0.1% Tween 20 in tris-buffered saline (TBS) and incubated with the appropriate antibodies (Supplementary Table 2) in 5% bovine serum albumin in TBS with 0.1% Tween 20 overnight at 4 °C. All primary antibody incubations were followed by incubation with secondary horseradish peroxidase (HRP)–conjugated antibody (Pierce) in 5% milk and 0.1% Tween 20 in TBS and visualized with SuperSignal West Pico or Femto Chemiluminescent Substrate (ThermoScientific), using a Biorad ChemiDoc MP imaging system.

Hematoxylin & Eosin (H&E) staining and Immunohistochemistry (IHC). Murine tumor tissues were fixed in 10% buffered formalin and embedded in paraffin. Sections of 6 μm were cut and deparaffinized. H&E staining was performed following standard procedures. For immunohistochemistry, after deparaffinization, antigen retrieval was performed using citrate buffer [10 mM Tris–sodium citrate, pH 6.0, 0.05% Tween 20] (for Neu1 and LAMPT), or 1 mM EDTA [10 mM Tris Base, 1 mM EDTA pH 9.0, 0.05% Tween 20] (for MyD, Myogenin and Adiponectin). Endogenous peroxidase was removed by incubation with 3% hydrogen peroxide in methanol for 5 min. For Neu1, LAMPT, and adiponectin, we used the ImmunPress HRP polymer system following the manufacturer’s instructions (VECTOR Laboratories cat# MP-7801) with overnight incubations with primary antibodies (Supplementary Table 2). Mouse anti-MyD and anti-Myogenin antibodies were biotinylated with the ARK (Animal Research Kit), Peroxidase (Dako K3954) following the manufacturer’s instructions. Signal detection was performed with the Vectastain Elite ABC HRP (Vector Laboratories Cat# PK6100) followed by Stable DAB chromogen (Invitrogen Cat#75018). Slides were counterstained with hematoxylin, dehydrated, and mounted with a xylene-based mounting medium. Formalin-fixed paraffin-embedded human RMS TMAs were obtained from the St Jude Pathology/Laboratory Medicine Department. Specimens were de-identified, and the study was approved by the Institutional Review Board (IRB approval Pro00008511) as non-human subject research. Human RMS TMAs were obtained from the St Jude Pathology/Laboratory Medicine Department (approved by IRB Pro00008511).

Masson’s Trichrome staining. Masson’s Trichrome staining was done as previously described20. In brief, FFPE (formalin-fixed paraffin-embedded) murine RMS samples and patient TMAs on slides were fixed in Bouin’s solution for 1 h at 60 °C. Sections were then stained sequentially at room temperature with Weigert’s iron hematoxylin, Biebrich scarlet-acid fuchsin, phosphotungstic/phosphomolybdic acid, and aniline blue. Sections were washed, dehydrated, and mounted with a xylene-based mounting medium. Trichrome Masson’s-stained slides were scanned to ×7 by adding 0.5 M NaHCO3. The Aperio color deconvolution and colocalization algorithms and the ImageScope software v12.4.3 (Leica Biosystems, Inc.) were used to quantify ECM/collagen (blue) deposition in the microenvironment and within tumor cells.

Nuclear morphometry analysis. H&E-stained murine RMS samples were scanned to ×20 scalable images with an Aperio ScanScope XT (Leica Biosystems, Inc.) and annotated using ImageScope software v12.4.3 (Leica Biosystems, Inc.). The Genie tissue classifier was trained to identify tumor nuclei and quantify their numbers and sizes.

Quantification of LAMPT staining. IHC stained images from RMS TMAs were scanned with a Panoramic 250 Flash III (3DHistech, Inc.) to ×2 to ×8 scalable images. Images from tissue cores from the whole slide were then manually annotated using HALO v3.2.1851.354 software (Indica Labs). A Membrane 5.1 algorithm was trained to identify LAMPT immunoreactivity at the membrane level. After positive immunoreactivity was identified with the algorithm, a density heatmap spatial analysis was performed on the analyzed samples to determine the minimal and maximal intercellular density and spacing of immune-positive neoplastic cells within a 25 μm radius in each core using the Spatial Analysis Module HALO 3.2 (Indica labs).

β-Hexosaminidase activity in tumor’s interstitial fluid. Frozen tumors were powdered in liquid nitrogen and homogenized in PBS for 2 min at 30 Hz in the Omni Prep Multi-Sample homogenizer (QIAGEN). Intestinal fluid was purified by sequential centrifugation steps at 300 × g for 10 min, 10,000 × g for 10 min, and 100,000 × g for 30 min to remove cells and cell debris. The supernatant/interstitial fluid was spun in an ultracentrifuge at 100,000 × g for 2 h (SW32Ti rotor) to remove small vesicles and exosomes. All steps were performed at 4 °C. Intestinal fluid was diluted 5 times in PBS and spun through a Sephadex column at pH 5.5 for 2 min at 850 × g. Beta-hexosaminidase activity was measured with 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide fluorometric substrate (3 mmol/L in citrate phosphate buffer pH 4.4 (6 mmol/L citric acid, 10 mmol/L disodium hydrogen phosphate) (Sigma M2133). Ten μl of intestinal fluid, or when needed a 1:10 dilution, was incubated at 37 °C with 10 μl of substrate for 1 h. The reaction was stopped with 200 μl carbonate stop buffer (0.5 M Na2CO3 with the pH set to 10.5), and the mixture was adjusted for dilution and length of incubation, and normalized to protein by BCA. The final units of activity were reported as nanomoles per hour per milligram.
Gene expression analyses. For microarray analysis of NPE and PE trunk and limb RMS, total RNA (100 ng) was converted into biotin-labeled cRNA (Ambion WT Expression Kit, Affymetrix Inc) and hybridized to Clariom S Mouse GeneChip (Affymetrix Inc) and signals summarized by RMA (Affymetrix Expression Console v1.1). Probe signals from arrays were normalized and transformed into log2 transcript expression values using the Robust Multiarray Average algorithm (Partek Genomics Suite v6.6). Patients’ RMS gene expression data from the Pediatric Cancer Genome Project (PCGP) were also used. For the comparison of murine versus human RMS, principal component analysis (PCA) and quality-control metrics removed outliers from both PCGP RNAseq and murine microarray data. We used data from 8 ARMS with PAX3/FOXO1 fusion, 4 ARMS with PAX7/FOXO1 fusion and 22 ERMS (accession# EGAS00001000256)71. Metadata were also supplemented for fusion calls from public RMS data (PeCan https://pecan.stjude.cloud/home). Correlations were calculated with Pearson’s correlation of deciles of the mean log2 FPKM (for RNAseq) and RMA values for the microarray’s expression by class. There were 91 genes that passed the false discovery rate (FDR) at 5%, having a difference of 5 or more deciles. GSEA [https://www.gsea-msigdb.org/gsea/index.jsp]72,74,75 was also performed using the curated pathways from MSigDB72,74. Differentially expressed transcripts were identified by ANOVA, and the FDR was estimated. Functional enrichment analysis of gene lists was performed using the DAVID bioinformatics databases (https://david.ncifcrf.gov/). Additional mentally derived gene sets, as well as established gene sets from MSigDB72,74. Normalized RMA microarray values were compared using voom-limma in R and displayed in a volcano plot.

Multiplex flow cytometry. NPE and PE tumors were dissociated with the Mouse Dissociation kit and gentle MACS Octo Dissociator with heaters following the manufacturer’s instructions (MACS, Miltenyi Biotec). Large aggregates persisting in the cell preparation were then removed by filtering them through 70 μm cell strainers. The resulting single-cell suspensions were stained with fluorochrome-conjugated monoclonal antibodies as noted in Supplementary Table 2, and analyzed using a BD FACSymphony A9 analyzer (BD, San Jose) equipped with 355, 488, and 640 nm lasers for excitation and an array of 30 detectors with the appropriate light filters for resolving the specified fluorochromes. Self-organizing maps for visualizing and interpreting cytometry data, FlowsOM, was used76 on 60,000 cells from limb and trunk NPE and PE RMS to generate density, heatmaps, and ISNE graphs. Cellular clusters were identified and annotated based on the differential expression of markers. Additional two-dimensional analyses were conducted using both FlowJo and FACS Diva software suites.

Statistics and reproducibility. Quantitative data are presented as mean ± s.d. of at least six biologically independent samples/tumors. A number of replicates are noted in the figure legends. Statistical analyses were performed using GraphPad Prism. Student’s t-test for unpaired and paired (two-tailed), Welch’s t-test for unpaired t-test, one-way ANOVA F test, Mann–Whitney for unpaired (two-tailed) t-test, all tests have a 95% confidence interval, and log-rank test for trend was performed to ascertain statistical significance and are noted in the figure legends. Mean differences were considered significant when P < 0.05. Source data underlying the main figures are presented in Supplementary Data 1.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The data that support the findings of this study are available from the corresponding authors on reasonable request. Uncropped and unedited blot images are provided in Supplementary Fig. 2. Microarray data in this publication are deposited in NCBI’s functional genomic data repository Gene Expression Omnibus (GEO) and are accessible through GEO series accession number GSE12378. Previously published datasets used in this study were deposited in the European Bioinformatics Institute (EMBL-EBI) and accessible through accession number EGAS00001000256. Source data underlying Fig. 1b, d, 1, j, 2–d, 3g, h, 6c, 7c–l and Supplementary Fig. 1a, 3a, b, d, e, 9a–c, 10a are provided within this paper (Supplementary Data 1). Patient’s RMS gene expression data can be accessed through MyData open resource page (PeCan https://pecan.stjude.cloud/home).

Received: 23 September 2021; Accepted: 8 September 2022; Published online: 20 September 2022

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