Epigenetic re-expression of HIF-2α suppresses soft tissue sarcoma growth

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In soft tissue sarcomas (STS), low intratumoral O2 (hypoxia) is a poor prognostic indicator. HIF-1α mediates key transcriptional responses to hypoxia, and promotes STS metastasis; however, the role of the related HIF-2α protein is unknown. Surprisingly, here we show that HIF-2α inhibits high-grade STS cell growth in vivo, as loss of HIF-2α promotes sarcoma proliferation and increases calcium and mTORC1 signalling in undifferentiated pleomorphic sarcoma and dedifferentiated liposarcoma. We find that most human STS have lower levels of EPAS1 (the gene encoding HIF-2α) expression relative to normal tissue. Many cancers, including STS, contain altered epigenetics, and our findings define an epigenetic mechanism whereby EPAS1 is silenced during sarcoma progression. The clinically approved HDAC inhibitor Vorinostat specifically increases HIF-2α, but not HIF-1α, accumulation in multiple STS subtypes. Vorinostat inhibits STS tumour growth, an effect ameliorated by HIF-2α deletion, implicating HIF-2α as a biomarker for Vorinostat efficacy in STS.
oft tissue sarcomas (STS) are a diverse group of malignancies arising from mesenchymal tissues, currently classified into ~50 distinct histological subtypes. Each year, 12,000 new cases are diagnosed in the United States, and roughly 4,000 succumb to this disease. While recent findings have defined molecular mechanisms underlying sarcomagenesis and disease progression, these cancers remain relatively understudied due to their varied clinical and pathological aetiologies, making effective treatment challenging. Current therapeutic options for localized disease include surgical resection, frequently in combination with radiation therapy and chemotherapy. For metastatic or unresectable STS, cytotoxic chemotherapy remains the primary approach; however, response rates are only 10–25% (refs 5,6). Therefore, it is critical to identify novel therapeutics, as well as biomarkers to predict their efficacy, to help improve patient outcomes.

Undifferentiated pleomorphic sarcoma (UPS), fibrosarcoma and dedifferentiated liposarcoma (DD-LPS) are undifferentiated high-grade sarcomas, which collectively represent up to 40% of newly diagnosed sarcomas in adults. UPS is among the most aggressive STS subtypes in adults, with a five-year survival rate of only 24% in patients with metastatic disease. Although UPS comprises 15% of newly diagnosed STS cases, its dedifferentiated phenotype suggests that it may represent a morphological end point for many other sarcomas. Further characterization may therefore provide broader insights into other aggressive STS subtypes.

One prominent feature of STS, including UPS, are severely hypoxic regions, a phenotype associated with lower overall survival rates. Cellular adaptation to hypoxic stress requires coordinated changes in gene expression, many of which are mediated by hypoxia-inducible factor (HIF)-1α and HIF-2α. Although HIF-1α and HIF-2α are stabilized under hypoxic conditions, extensive data indicate that many important HIF targets are controlled specifically by one isoform or the other. Additionally, the impact of HIF-α isoform stabilization is context-dependent, as they have been demonstrated to promote or suppress tumour growth in different cancers. Several HIF inhibitors have been developed for clinical intervention, and while certain compounds demonstrate isoform-specific inhibition, many affect both HIF-1α and HIF-2α equally. Thus, the role of both HIF-α subunits in specific tumour contexts must be characterized before using either pan or isoform-specific HIF-α drugs.

Whereas HIF-1α has recently been shown to promote metastasis in UPS and fibrosarcoma, the role of HIF-2α in STS has not been established. In this study, using a genetically engineered UPS mouse model that faithfully recapitulates human disease, as well as fibrosarcoma and liposarcoma xenografts, we found that HIF-2α expression surprisingly suppresses tumourigenesis. Loss of HIF-2α (encoded by the Epas1 gene) increased sarcoma tumour cell proliferation. Additionally, RNA-seq analysis indicated that Anoctamin-1 (ANO1, DOG1 and TMEM16A) encoding a calcium-activated chloride channel, was expressed at elevated levels in HIF-2α deficient autotchonous UPS tumours relative to controls. In turn, ANO1 overexpression coincided with elevated CAMKII and mTORC1 signalling in these tumours. mTORC1 senses nutrient availability, and regulates cellular growth, biosynthetic activity and ribosomal biogenesis; as such, disorganization of this pathway occurs in a...
variety of cancer types, including sarcomas\textsuperscript{30}. Decreased EPAS1 messenger RNA (mRNA) expression (with no copy number variation) was detected in the majority of STS patient samples analysed, including UPS, fibrosarcoma and liposarcoma. These data suggest that EPAS1 expression is suppressed by epigenetic mechanisms in multiple sarcoma subtypes. Altered epigenetics have been observed in many cancers, with dysregulation of the epigenome proposed as an important mechanism whereby tumours progress\textsuperscript{31}. Of note, treatment with the chromatin modifying agent suberanilohydroxamic acid (SAHA, Sigma-Aldrich, St Louis, MO, USA; Vorinostat), a clinically approved histone deacetylase inhibitor (HDACi)\textsuperscript{32}, significantly increased HIF-2α expression in several STSs, and inhibited growth in a HIF-2α dependent manner.

Figure 2 | Loss of HIF-2α promotes liposarcoma and fibrosarcoma tumour growth in vivo. (a) EPAS1 mRNA expression from Oncomine analysis of the Barretina et al. sarcoma patient samples data set\textsuperscript{34}. Values are normalized to median-centered intensity, and shown on a log₂ scale. Dediff. lipo., dedifferentiated liposarcoma; MFH/myxofibro., myxofibrosarcoma; MFH/pleo., UPS; myxoid/RC lipo., myxoid/round cell liposarcoma; pleomph. lipo., pleomorphic liposarcoma. (b) Kaplan–Meier curve of overall survival of liposarcoma patients from the Gobble et al. data set\textsuperscript{47}) compared with dedifferentiated liposarcoma ('DD-LPS', n = 20) patient samples from the Gobble et al. data set. Values are normalized to median-centered intensity, and shown on a log₂ scale. ***P<0.001. (d) Left: tumour volume of LPS246 liposarcoma xenografts with scrambled (SCR; n = 9) or HIF-2α shRNA (n = 9). Right: immunoblot demonstrating HIF-2α knockdown with two independent HIF-2α shRNAs compared with SCR shRNA. **P<0.01. (e) Left: weights of LPS246 xenograft tumours with SCR shRNA (shSCR) or HIF-2α shRNA (shHIF-2α 2) at time of killing, measured in grams. **P<0.01. Right: image of representative LPS246 tumours with shSCR or HIF-2α shRNA (shHIF-2α 2). (f) Left: tumour volume of HT-1080 fibrosarcoma xenografts with SCR (n = 5) or HIF-2α shRNA (HIF-2α 1; n = 10). Right: immunoblot demonstrating HIF-2α knockdown with HIF-2α shRNA (HIF-2α 1) compared with SCR shRNA. **P<0.01. (g) Weights of HT-1080 xenograft tumours with shSCR and HIF-2α shRNA (shHIF-2α 1) at time of killing, measured in grams. *P<0.05. All error bars represent the mean ± s.e.m. P-values for part c–g were calculated using a two-tailed Student’s t-test.
Results

HIF-2α suppresses tumour growth in UPS. To address the role of HIF-2α in STS, we crossed the previously described autochthonous KP' (LSL-KrasG12D/+; Trp53fl/fl) UPS mouse strain with Epsafl/fl; Ano1mice3 to generate 'KPH2' (LSL-KrasG12D/+; Trp53fl/fl; Epsafl/fl) animals. Hind limb UPS tumours are generated by injection of adenovirus expressing Cre-recombinase (Ad-Cre) into the gastrocnemius muscle. Of note, KPH2 mice formed UPS lesions at a significantly elevated rate compared with KP control animals, with 50% of KP and KPH2 strains exhibiting palpable tumours 56 and 47 days, respectively, after Ad-Cre injection (Fig. 1a). PCR analysis confirmed efficient EpsaI deletion in KPH2 tumours (Supplementary Fig. 1a). Both KP and KPH2 samples displayed a similar heterogeneous, multimucated appearance consistent with UPS and local muscle invasion was also observed, albeit more extensively in the KPH2 than KP tumours (Supplementary Fig. 1b). In agreement with prior reports25, KP and KPH2 tumours exhibit areas of localized hypoxia, as demonstrated by Hypoxyprobe staining (Supplementary Fig. 1c). Interestingly, KPH2 tumours were larger than KP controls, with significantly increased mass at 7 weeks post-Ad-Cre injection (Fig. 1b). KPH2 tumours were also more proliferative than KP, as demonstrated by BrdU uptake (Fig. 1c), while apoptotic rates were unchanged based on cleaved caspase-3 levels (Supplementary Fig. 1d).

Although HIF-1α and HIF-2α have unique transcriptional targets, they also regulate common genes in a coordinate or even opposing manner16–18. To rule out the possibility that the effects on mTORC1 signalling in soft tissue sarcomas.

HIF-2α loss promotes DD-LPS and fibrosarcoma growth. To determine whether HIF-2α expression was decreased in other STS subtypes, we first screened publicly available microarrays of human STS using Oncomine. Analysis of a data set recently published by Barretina et al.34 showed that EPAS1 mRNA expression was lower in fibrosarcoma, leiomyosarcoma, liposarcoma and UPS (previously named malignant fibrous histiocytoma) patient samples compared with normal adipose tissue (Fig. 2a). Additionally, liposarcoma and UPS tumours demonstrated decreased immunostaining for HIF-2α protein compared with normal artery and skeletal muscle tissue (Supplementary Fig. 2a). In a separate data set35, decreased EPAS1 mRNA expression correlated with worse overall survival in liposarcoma patients (Fig. 2b). Of note, EPAS1 levels were significantly lower in more aggressive liposarcoma subtypes, such as dedifferentiated and pleomorphic liposarcoma (Fig. 2c and Supplementary Fig. 2b), and in this data set, the bottom 50% EPAS1 expressing cohort have a higher risk of death (relative risk of 6.67) compared with the top 50% EPAS1 expressing cohort. Collectively, these analyses indicate EPAS1 expression is decreased in multiple STS subtypes, and lower EPAS1 levels correlate with poorer prognosis in a cohort of liposarcoma patients.

To test the functional effects of HIF-2α suppression in different STS subtypes, we inhibited HIF-2α using two independent shRNAs in LPS246 liposarcoma cells, which resulted in increased xenograft volume growth and mass (Fig. 2d,e, Supplementary Fig. 2c). Furthermore, HIF-2α depletion promoted growth of HT-1080 fibrosarcoma tumour xenografts in vivo (Fig. 2f, Supplementary Fig. 2d). Importantly, HIF-1α ablation did not affect tumour growth (Supplementary Fig. 2d), consistent with previous reports25. These results demonstrate that inhibition of HIF-2α in vivo accelerates growth of multiple STS, including UPS, fibrosarcoma and liposarcoma.

Figure 3 | Loss of HIF-2α increases mTORC1 signalling in STS. To define mechanisms whereby HIF-2α opposes sarcomagenesis using an unbiased approach, we performed RNA-seq analysis of KP (n = 3) and KPH2 (n = 4) tumours. By principle component analysis, KP and KPH2 tumours segregated into distinct populations (Supplementary Fig. 3a). Gene set enrichment analysis of differentially expressed genes revealed a strong enrichment for those regulating RNA polymerase I promoter opening and transcription in KPH2 compared with KP samples (Fig. 3a and Supplementary Fig. 3b), suggesting increased ribosome biogenesis in the KPH2 tumours. As c-MYC and mTORC1 signalling promote proliferation and ribosome biogenesis in cancers29,31, we assessed whether either pathway was more active in KPH2 tumours compared with KP controls. KPH2 tumours displayed increased c-Myc protein levels on average, but only modest changes in the expression of several canonical c-MYC target genes (Myc, Cnd1, Cdn2, Mm5 and Cdkn1a).
Supplementary Fig. 3c) were observed (for more discussion on this matter, see below). Additionally, Ingenuity Pathway Analysis (IPA) did not predict c-MYC activation in KPH2 tumours. In contrast, IPA predicted increased mTOR activity based on differentially expressed mTOR targets between KP and KPH2 tumours (Supplementary Fig. 3d). The canonical mTORC2 target, phosphorylated AKT at serine 473, was not significantly different between KP and KPH2 tumours (Fig. 3b)\textsuperscript{37}. However, using a Fischer’s exact test, we determined that KPH2 tumours had a statistically significant mTORC1 target gene signature, for targets predicted to be both elevated ($P = 0.014$) and suppressed ($P = 0.010$) by mTORC1 (ref. 38). Canonical mTORC1 signalling outputs, phosphorylated 4E-BP1 and S6K1 (ref. 39), were also assessed in KP and KPH2 tumours, and elevated 4E-BP1 phosphorylation was observed in KPH2 tumours compared with KP (Fig. 3b). We perceived variable increases in S6K1 phosphorylation in whole KP and -KPH2 UPS lysates, indicating that mTORC1 activity may vary in tumour subdomains. Therefore, we examined phosphorylated-S6 staining in tumour parenchyma by IHC, and noted significantly
expanded areas of phosphorylated-S6 in KPH2 (n = 5) compared with KP (n = 5) sections (Fig. 3c). Phosphorylated 4E-BP1 levels were elevated in KPH2 tumour-derived cells as compared with KP tumour-derived cells (Supplementary Fig. 3e), with the expected increase in unphosphorylated 4E-BP1 under hypoxic growth conditions (Fig. 3d). Consistent with this finding, proliferation was also increased in KPH2 cells (Supplementary Fig. 3f). Similar increases in phosphorylated 4E-BP1, S6K1 and S6 were apparent in HIF-2α knockdown LPS246 xenografts (Fig. 3e,f). Although some heterogeneity exists in tumours expressing or lacking HIF-2α, taken together, these data indicate that mTORC1 activity is increased in HIF-2α deficient sarcomas.

To elucidate the mechanisms whereby HIF-2α alters mTORC1 activity in UPS, we examined the top differentially expressed genes base on RNA-seq data. Several targets identified were related to skeletal muscle and extracellular matrix (for example, Lama5, Col18a1 and Dmnp1), consistent with KPH2 showing more local invasion into the adjacent skeletal musculature (Supplementary Fig. 1b and 4a). However, Ano1 (encoding TMEM16A, Anoctamin-1 and DOG1) transcripts were significantly increased in KPH2 tumours relative to controls (Fig. 3g). Ano1, a calcium-activated chloride channel28, is overexpressed in a variety of cancers, including gastrointestinal stromal tumours, breast cancer, and head and neck cancers40–42. KPH2 tumours also exhibited elevated ANO1 protein levels compared with KP tumours (Fig. 3h), although the available antibody exhibited relatively weak immunoreactivity with both KP and KPH2 tumour lysates.

ANO1 has previously been reported to promote both EGFR and CaMKIIα signalling in breast cancer cell lines42. While we failed to observe a consistent pattern of increased EGFR phosphorylation in KPH2 tumours (Fig. 3h), these tumours expressed higher levels of phosphorylated CaMKIIα. These data are consistent with IPA analyses, which predicted increased calcium signalling in KPH2 tumours (Supplementary Fig. 4b). Since intracellular calcium and CaMKIIα have been shown to enhance mTORC1 signalling43,44, we hypothesized that increased mTORC1 activity in KPH2 tumours was mediated through ANO1 and CaMKIIα signalling. To test this, we serum starved KP and KPH2 tumour-derived cells for 24 h, and then treated with replete media plus dimethyl sulfoxide (DMSO) or CaCCinh-A01, a small molecule inhibitor of ANO1 activity42. KPH2 cells maintained slightly higher levels of ANO1 compared with KP in vitro (Supplementary Fig. 4c), although this phenotype is more striking in tumours as they were cultured under normoxic conditions. As expected, CaCCinh-A01 treatment reduced phosphorylated CaMKIIα levels more effectively in KPH2 compared with KP cells (Fig. 3i). Moreover, CaCCinh-A01 diminished mTORC1 activation (Fig. 3i), consistent with our findings that ANO1 is more highly expressed in KPH2 cells. In addition, CaCCinh-A01 treatment decreased cell proliferation more potently in KPH2 than KP cells after 3 days of treatment (Fig. 3i). To determine if ANO1 inhibition diminished HIF-2α deficient sarcoma growth in vivo, Ano1 targeted shRNAs were introduced into KP and KPH2-derived UPS cells (Supplementary Fig. 4d,e) to generate allografts. Similar to our in vitro data, inhibition of ANO1 reduced tumour growth in KPH2 but not KP allografts (Fig. 3j). Collectively, these data suggest that loss of HIF-2α increases ANO1 accumulation, activating CaMKII and mTORC1 signalling in UPS tumours, enhancing their growth (see below for further discussion).

HDAC inhibition increases EPAS1 expression in UPS. Having determined that HIF-2α exhibits a tumour suppressive role in UPS and other STs, we examined possible mechanisms whereby HIF-2α is silenced in these lesions. Initially, we analysed Epas1 expression over time in the KP tumour model. One cohort of animals was euthanized 7 weeks post-Ad-Cre injection (‘KP early’, n = 7), and another at 8.5 weeks post-Ad-Cre injection (‘KP late’, n = 9). Epas1 mRNA levels in the KP late cohort were significantly diminished compared with KP early, with Epas1 mRNA of mouse skeletal muscle shown as a comparison (Fig. 4a). Therefore, we concluded that Epas1 expression is lost progressively over the course of sarcomagenesis. Analysis of TCGA sarcoma patient samples revealed that 25% of all human sarcomas have lost at least one copy of EPAS1, while 67% remain diploid (Supplementary Fig. 5a). As EPAS1 mRNA levels are consistently diminished in several STS subtypes (Fig. 2a), we hypothesized that EPAS1 is epigenetically silenced in a significant fraction. Using a publically available data set of human sarcoma cell lines treated with various cancer therapeutics, we identified a significant correlation between EPAS1 mRNA abundance and sensitivity to the HDACi Vorinostat, also known as SAHA (Fig. 4b, Supplementary Fig. 5b)45. In contrast, no such correlation existed between HIF1A expression and SAHA sensitivity in the same cell lines (Fig. 4c). We also analysed DNA methylation along the EPAS1 locus in the TCGA sarcoma data set, and found no consistent methylation changes in the samples (Supplementary Table 1). Together, these results suggest that HDACs specifically suppress EPAS1 in sarcoma, and that re-expressing EPAS1 could be a mechanism underlying HDACi’s inhibition of sarcoma growth.

To test whether HDACi could activate EPAS1 expression in sarcoma, two independent mouse UPS cell lines, derived from KP tumours (KP230 and KP250), were treated with multiple SAHA concentrations (500 nM–2 μM) in vitro. SAHA exposure increased Epas1 mRNA levels (Fig. 4d), an effect also observed in HT-1080 cells (Supplementary Fig. 5c). Importantly, at the highest dose tested (2 μM), SAHA treatment had no effect on HIF1A mRNA in UPS or HT-1080 cell lines (Fig. 4e and Supplementary Fig. 5c). Consistent with these findings, 2 μM SAHA also increased HIF-2α protein levels in both KP230 and KP250 cells, while HIF-1α protein was unchanged (Fig. 4f). SAHA treatment also resulted in elevated mRNA levels of Serpine1, a HIF-2α target46, at all concentrations tested (Fig. 4g).

We also investigated whether altering DNA methylation would impact EPAS1 expression, given that DNA methylation regulates EPAS1 in renal cell carcinoma47. However, treatment with 5-azacytidine, a DNA methyltransferase inhibitor48, and 3-Deazaneplanocin A (Cayman Chemicals, Ann Arbor, MI, USA), an EZH2 histone methyltransferase inhibitor, failed to significantly increase Epas1 mRNA levels in UPS cell lines (Supplementary Fig. 5d). To rule out off-target effects of SAHA on EPAS1, we treated KP230, KP250 and HT-1080 cells with an independent class I/II HDACi, Trichostatin A (Supplementary Fig. 5e). Trichostatin A increased Epas1 and Serpine1 mRNA levels, but had no effect on Hif1a mRNA expression. However, treatment with nicotinamide, a class III HDACs/sirtuins inhibitor49, did not change Epas1 or Hif1a mRNA levels in the same cell lines (Supplementary Fig. 5f). Finally, chromatin immunoprecipitation (ChIP) was performed to verify that SAHA exposure increased the abundance of acetylated histones at the Epas1 locus. Using available ChIP-seq data from the UCSC genome browser of mouse liver and heart tissue, primers targeting the region 500 bp upstream of the Epas1 transcription start site (TSS) were designed. ChIP-seq/RNA-seq analyses of EPAS1 expressing tissues indicated this region is rich in histone acetylation. Treatment with 2 μM SAHA increased levels of acetylated histone H3 near the Epas1 TSS compared with DMSO control under normoxic (21% O2) and hypoxic (0.5% O2) conditions (Fig. 4h). Conversely, no increases in acetylated histone H3 were observed.
histone H3 at 500 bp upstream of the Hif1a TSS were detected. In aggregate, these results indicate that HIF-2α expression is lost during UPS disease progression, and epigenetic therapeutics like SAHA specifically elevate HIF-2α, but not HIF-1α, levels.

**SAHA slows UPS growth in a HIF-2α dependent manner.** Findings described above suggest that SAHA treatment may suppress sarcoma cell proliferation and tumour growth. We first tested this hypothesis by investigating SAHA’s ability to inhibit UPS, HT-1080 and LPS246 sarcoma cell proliferation in vitro. Of note, SAHA treatment significantly decreased cell growth under both normoxia and hypoxia (1% O2; Fig. 5a, Supplementary Fig. 6a,b).

Next, we evaluated SAHA’s therapeutic efficacy against UPS allografts in vivo, and found that SAHA administration (50 mg kg−1 per day) significantly reduced tumour growth (Fig. 5b), and final tumour weights (Fig. 5c). Importantly, no obvious adverse effects of treatment were observed, and the mice maintained weight similar to DMSO-treated controls (Fig. 5d). Surprisingly, Epas1 mRNA levels were only slightly elevated in the SAHA-treated tumours compared with controls, although there was no change in Hif1a levels, as expected (Supplementary Fig. 6c). We suspected that KP tumour cells developed resistance to SAHA, and its effect on Epas1 re-expression, over the extended course of the experiment (8 days). Consistent with this hypothesis, tumours from UPS-bearing mice treated with SAHA for only 4 days displayed significantly increased Epas1 mRNA levels (Supplementary Fig. 6d). As SAHA can affect multiple cellular targets and processes50, we assessed the specific role of HIF-2α in SAHA-mediated anti-tumour effects. Two independent shRNAs, one producing a partial inhibition (H2α shRNA 1), and a second more effective shRNA (H2α shRNA 2), were used to deplete HIF-2α in KP250 cells (Fig. 5e, Supplementary Fig. 6e). Both HIF-2α shRNAs inhibited
SAHA-induced *Epas1* and *Serpine1* mRNA re-expression in UPS cells. Mice bearing in vivo UPS allografts expressing control or HIF-2α shRNAs were then treated with DMSO or SAHA (50 mg kg⁻¹ per day) once tumours had reached 100 mm³ (10 days post-implantation). SAHA significantly slowed the growth of control tumours, while HIF-2α inhibition abrogated this effect in a dose-dependent manner (Fig. 5e). Conversely, overexpression of HIF-2α in HT-1080 fibrosarcoma cells inhibited cell proliferation in vitro under hypoxic conditions (Supplementary Fig. 6f,g). When treated with SAHA, we observed a further increase in HIF-2α protein in empty vector and HIF-2α overexpressing cells, and a further decrease in cell proliferation likely due to increased expression of the endogenous HIF-2α mRNA (Supplementary Fig. 6g,h).

The standard chemotherapeutic approach for unresectable STS is doxorubicin. To demonstrate that SAHA treatment could be safely and effectively incorporated into the clinical setting, we investigated the efficacy of combining doxorubicin and SAHA.
Figure 6 | HIF-2α expression is required for SAHA’s efficacy in an autochthonous UPS model. (a) Top: schematic of tracking autochthonous KP tumour growth in DMSO- and SAHA-treated mice. After Ad-Cre injection, mice were imaged bi-weekly until tumours were detectable and measured to 50–100 mm³. Mice were randomized to DMSO control (n = 5) or SAHA (n = 5; 50 mg kg⁻¹ per day) treatments, and tumour growth was followed by bi-weekly CT scans. Bottom: representative axial CT images of KP mice pre- and post-treatment with DMSO or SAHA. Dashed white line demarcates the tumour boundary. (b) Relative sizes of individual tumours from KP mice receiving DMSO or SAHA treatment. (c) Comparison of the relative sizes of all DMSO- and SAHA-treated KP tumours at the time of killing (error bars are ± s.e.m.). *P < 0.05. P-values were calculated from a two-tailed Student’s t-test. (d) Weights of KP mice pre-treatment and at time of killing (error bars are ± s.e.m.). Black squares, DMSO-treated mice; red circles, SAHA-treated mice. (e) Immunoblot of HIF-2α protein in KP autochthonous tumours from DMSO- and SAHA-treated mice. GAPDH served as loading control. (f) Left: representative transverse CT images of KPH2 mice pre- and post-treatment with DMSO or SAHA. Mice were treated and imaged as described in a. Right: relative sizes of individual tumours from KPH2 mice receiving DMSO (n = 4) or SAHA (n = 5) treatment. (g) Comparison of the relative sizes of all DMSO- and SAHA-treated KPH2 tumours at the time of killing (error bars are ± s.e.m.). (h) Model of EPAS1 regulation in the STS examined. EPAS1 expression is epigenetically downregulated through class I/II HDACs. Loss of HIF-2α increases ANO1 and calcium signalling, which subsequently increases mTORC1 activity in tumours and promotes sarcoma proliferation. HIF-2α loss may also increase sarcoma growth independent of this pathway.
Although combination therapy reduced UPS allograft growth in vivo compared with DMSO or doxorubicin alone (Fig. 5f), this inhibition was similar to SAHA treatment alone (Fig. 5e). Moreover, HIF-2α inhibition in these allografts fully abrogated this effect. (Fig. 5g,h). Collectively, these data strongly suggest that SAHA limits UPS proliferation in vitro and in vivo, in a HIF-2α-dependent manner.

We next evaluated the efficacy of SAHA in an autochthonous STS tumour model, using KP mice. After initiating tumours with Ad-Cre injection, we performed bi-weekly CT scans of the animals’ lower limbs. When tumours reached 50–100 mm³, animals were treated with DMSO or SAHA (50 mg kg⁻¹ day⁻¹) daily (Fig. 6a). SAHA decreased relative tumour growth over time, compared with controls (Fig. 6b), resulting in significantly reduced relative tumour size by day 10. (Fig. 6c). Total animal weight did not change due to SAHA treatment (Fig. 6d).

Importantly, HIF-2α protein levels increased in SAHA-treated tumours compared with controls (Fig. 6e). SAHA also decreased KP tumour cell proliferation, as indicated by reduced Ki67 tumours compared with controls (Fig. 6e). SAHA also decreased mTORC1 activation. Importantly, further work will be elucidated. Importantly, ChIP-seq data assembled in the UCSC genome browser show Myc/Max binding near the promoter region of ANO1 in breast and leukaemia cells. Thus, one possible explanation for our findings is that Myc, whose level is elevated in KPH2 tumours (Supplementary Fig. 7a) with no concomitant change in apoptosis (Supplementary Fig. 7b). Importantly, SAHA’s effects on tumour growth (Fig. 6f,g) and proliferation (Supplementary Fig. 7c,d) were completely abrogated in KPH2 tumours, underscoring the importance of HIF-2α in SAHA-mediated anti-tumour effects.

Discussion

Current chemotherapeutic approaches for the treatment of unresectable and metastatic STS have low-response rates. One major obstacle to developing better treatment regimens has been the myriad of unique subtypes with distinct genetic alterations, coupled with the relatively low incidence of these malignancies. Until recently, clinical trials often combine patients with diverse STS histologic subtypes, which are induced by heterogeneous genetic alterations potentially from multiple cells of origin, into a single study. Our growing understanding of context specificity in tumours, particularly in sarcomas, has shown that this approach has many drawbacks. Newer targeted therapies are increasingly being investigated, not only in a sub-type specific manner, but also based on the molecular underpinnings of the tumours. It will be very beneficial to identify therapeutic biomarkers that span multiple subtypes and determine treatment strategies based on gene expression changes that are linked to drug sensitivity. Identification of biomarkers that predict drug response should allow clinicians to better select patient populations most likely to respond to treatment. Although these biomarkers are scarce in STS, we report here that HIF-2α expression levels may be particularly effective for this purpose.

Clinical data have identified intratumoural hypoxia and HIF-1α as one of the most important prognostic factors in metastatic potential of STS. Consistent with this finding, we have shown previously that HIF-1α enhances tumour metastasis through modifications of the collagen network in UPS and fibrosarcoma. Conversely, we demonstrate here that HIF-2α expression opposes UPS, fibrosarcoma and liposarcoma growth in vivo, decreasing tumour cell proliferation and inhibiting mTORC1 activity. Although tumours exhibit significant heterogeneity in our in vivo models, both within a given sample and between samples, we saw significant enrichment of mTORC1 activation. Importantly, further work is necessary to determine if this mechanism is shared with other STS subtypes not examined here. It is noteworthy that low HIF-2α expression is correlated with lower overall survival rate in a cohort of liposarcoma patients. Additionally, loss of HIF-2α did not alter metastatic potential in UPS in vitro or in vivo. Although mTORC2 signalling has been shown to promote HIF-2α accumulation in cancers like renal cell carcinoma, our results suggest that HIF-2α inhibits mTORC1 signalling in at least the high-grade STS subtypes examined. This context dependency of HIFs and mTOR signalling in cancer warrants further study. For UPS tumours, loss of HIF-2α increases ANO1, promoting GαsMI and mTORC1 activity (Fig. 6h). Interestingly, a previous study demonstrated that HIF-1α inhibition increased ANO1 mRNA expression in renal cysts; yet we are the first to connect HIF-2α loss with increased ANO1 in UPS. However, HIF-2α may also inhibit sarcoma growth in an ANO1 and mTORC1-independent fashion (Fig. 6h). Given the multitude of targets and biological processes HIF-2α controls, the mechanism whereby HIF-2α inhibition increases ANO1 levels remains to be elucidated. Importantly, ChIP-seq data assembled in the UCSC genome browser show Myc/Max binding near the promoter region of ANO1 in breast and leukaemia cells. Thus, one possible explanation for our findings is that Myc, whose level is elevated in KPH2 tumours (Supplementary Fig. 7a) with no concomitant change in apoptosis (Supplementary Fig. 7b). Importantly, SAHA’s effects on tumour growth (Fig. 6f,g) and proliferation (Supplementary Fig. 7c,d) were completely abrogated in KPH2 tumours, underscoring the importance of HIF-2α in SAHA-mediated anti-tumour effects.

Given that HIF-2α expression opposes STS tumourigenesis, we sought a class of compounds that could induce re-expression of HIF-2α in sarcoma cells, and found that the FDA-approved HDACi SAHA (Vorinostat) reactivates EPAS1 expression in STS cells, inhibiting sarcoma progression (Fig. 6h). HDACi have been assessed in vitro and in vivo pre-clinically for the treatment of a variety of malignancies, including specific sarcoma subtypes such as malignant peripheral nerve sheath tumours and synovial sarcoma, with some early success. Clinical trials testing chemotherapy with HDACi in STS are still ongoing, as well as newer generations of HDACi, such as abexinostat, with potentially improved anti-tumour effects and pharmacokinetics. To our knowledge this study is the first to show HDACi treatment reduces growth of autochthonous UPS tumours in vivo, and demonstrate that HIF-2α re-expression is required for SAHA’s anti-proliferative effects in the context of UPS.

Importantly, combination of standard doxorubicin, a first-line chemotherapeutic against STS, with vorinostat (or newer generation HDACi such as abexinostat) could be a suitable treatment strategy that has exciting clinical potential. These results suggest that HDACi therapies will be most effective against sarcomas that have epigenetically suppressed HIF-2α expression, and not genomic deletion of the locus. HIF-2α may therefore serve as an important biomarker for clinicians designing future clinical trials with HDAC inhibitors in sarcoma patients.
Methods

Cell culture. HT-1080 (fibrosarcoma) and HEK-293T cell lines were purchased from ATCC (Manassas, VA, USA). KP210, KP230 and KP250 cell lines were derived from UPS mouse tumours25. These high-grade neoplasms showed myofibroblastic differentiation and atypical nuclei27, and by expression profiling matched human MFH/UPS (ref. 26). LPS246 was established from a primary dedifferentiated liposarcoma sample (confirmed with MDM2 FISH analysis), and was provided by Dr Dina Lev (Core Facilities, MD Anderson Cancer Center, Houston, TX)63. All cell lines were confirmed to be negative for mycoplasma contamination.

Drug treatment in vitro and lentiviral transduction. For in vitro drug studies, SAHA and Trichostatin A (Sigma-Aldrich) were dissolved in DMSO, and diluted in media to final concentrations of 500 nM–2 μM and 250–15.6 nM, respectively. Nicotinamide (Sigma-Aldrich) was dissolved in dH2O and diluted in media to a final concentration of 5 μM. CaCCInh-A01 (Sigma-Aldrich) was dissolved in DMSO and diluted in media to final concentrations of 1–2 μM. Every 48 h, media with fresh drug was changed during each experiment.

For short hairpin RNA (shRNA)-mediated knockdown, the PKLo.1 background vector was used (GE Life Sciences, Mickleton, NJ, USA). shRNA plasmids were packaged using a third-generation lentiviral system (pMDL, VSV-G and pRSV-REV), and expressed in HEK-293T cells. Human EPAS1 TRCN: 000038305, 000038307, Mouse EPAS1 TRCN: 000082236, 000082307, Mouse ANO1 TRCN: 000173335, 000193423. Scrambled shRNA was obtained from Addgene (Cambridge, MA, USA). Viral supernatant was collected 24 h post-transfection, and concentrated using 10-kDa Amicon Ultra-15 centrifugal filter units (Millipore, Billerica, MA, USA). For re-expression studies, human HIF-2α (ref. 18) in the right hind limb muscle as previously described27. KP, KPH2 and KPA mice were killed either 7 weeks after Ad-Cre injection, or when tumours reached the maximal permissible size. Mice were injected with BrdU 30 min before killing. For HT-1080 xenografts, 2×10⁶ cells were injected into the peritoneal cavity (50 mg kg⁻¹ body mass). To accurately measure tumour mass, the weights and measurements were confirmed by a board-certified radiologist who was blinded to the treatment used for each mouse.

Immunohistochemistry and immunofluorescence. Immunohistochemistry and immunofluorescence were performed on 5-μm paraffin embedded tissue sections. For immunohistochemistry, enzymatic Avidin-Biotin Complex-diamino-benzidine staining (Vector Labs) was used with haematoxylin used for counterstaining nuclei according to standard protocols. Immunohistochemistry was performed on soft tissue sarcoma and normal arterial and skeletal muscle tissue array (US Biomax, Rockville, MD, USA #S0801a). The following primary antibodies and concentrations were used for immunohistochemistry: anti-BrdU 1:40 (Abcam #ab6326), anti-cleaved caspase-3 1:300 (Cell Signaling #9661) anti-Ki67 1:100, anti-phospho-HIF-2α (ThermoPierce #PA1-16510). The following primary antibodies and concentrations were used for immunofluorescence: anti-CDC1 1:50 (Abcam #ab23634), anti-pimonidazole FITC 1:100 (Hypoxyprobe HP2). Mounting media with DAPI Life Technologies #P36930 was applied and images were imaged using a Leica DMRB microscope and an Olympus DP72 camera.

Quantitative reverse transcriptase-PCR. Total RNA was isolated from cells using the TRIzol reagent protocol (Invitrogen) and from tumours and skeletal muscle tissue using the RNAeasy minikit (Qiagen). RNA was reverse transcribed using the High-Capacity RNA-to-cDNA kit (Applied Biosystems), and transcript expression was determined by quantitative PCR using the Applied Biosystems Viia7 system. Target cDNA levels were measured with Taq-Man primer/probe sets (Applied Biosystems) for the following mouse and/or human targets: EPAS1 (human: HS01261124_M1, mouse: MM00432359_M1), CCND1 (mouse: MM04033807_M1), MCM5 (mouse: MM01243769_M1), CDKN1A (mouse: MM00234428_M1), HPRT1 (human: HS01032628_G1, mouse: MM01318743_M1) and ACTB (human: HS00609665_G1, mouse: MM00467939_S1). Expression levels were normalized to HPRT1 and ACTB.

Proliferation assays. Cells were plated in triplicate for each data point, and incubated overnight in tissue culture dishes. The following day, SAHA or DMSO, dilute in growth media, was added to the cells, and the cells were either in normoxic conditions (21% O₂) or hypoxic conditions (1% O₂) using a Ruskinn InVivO2 400 workstation. Media with drug was changed every 2 days. Cells were trypsinized, resuspended in PBS and counted using a haemocytometer on the days indicated.

Chromatin immunoprecipitation. ChIP assays were performed using QuickChIP assay kits (Novus Biologicals). Briefly, five million KP250 cells were fixed in 1% formaldehyde for 10 min at room temperature. The crosslinking reaction was stopped by glycine. Cells were washed, collected and lysed using SDS buffer containing protease inhibitor cocktail, and then sonicated into DNA fragments of 300–800 bp in size. Supernatants were

Protocol. Cells were pelleted down, and washed with DMEM/F-12. Cells were cultured in DMEM (Cellgro, Manassas, VA, USA) supplemented with 10% foetal bovine serum and 2% L-glutamine. The media was changed every 2 days. When cells reached 80%–90% confluence, the media was changed to 0.53 μM HIF-2α-shRNA. No inclusion/exclusion criteria were put in place. The experiment was performed according to National Institutes of Health guidelines and approved by the University of Pennsylvania Institutional Animal Care and Use Committee. At the time the studies were performed on KP, KPH2 and KPA animals were concluded. The University of Pennsylvania’s Animal Care and Use Committee’s guidelines on tumour load assays specified that implanted or autochthonous tumours in aggregate should not exceed 10% of the animal’s body weight. All mice weighed >25 g each, and tumour weights for both the KPH2 and KPA cohorts were recorded as 0.2–2.2 g (that is, <10% body mass). To accurately measure tumour mass, the weights of contralateral control legs for each mouse were compared with legs-bearing tumours (Fig. 1b,d). Sample size for each experiment was estimated using the formula n = (2zx²/σ²)E², with z = 0.05, and σ and E based off of initial HT-1080 xenograft experiments with scrambled and HIF-2α shRNA. No inclusion/exclusion criteria parameters were used in our studies. All tumours and animals used in experiments were included in data analysis.

Tumour cell isolation. KP and KPH2 tumour cells were isolated from primary KP and KPH2 UPS tumours, respectively. Briefly, UPS were harvested from mice, and transferred to a cell-culture hood and cut into tiny pieces on a petri dish. Tumours were digested using collagenase (StemCell #07902, Vancouver, Canada) for 1 h at 37°C with 1 mg ml⁻¹ of Dr Dimonide (Sigma-Aldrich) with 1 mg ml⁻¹ of papain added, and the digested mixture was filtered through a 40-μm filter. Red blood cells were lysed with ACK lysis buffer (Lonza, Allendale, NJ) according to the manufacturer’s
diluted and pre-cleared with salmon sperm DNA/protein A/G agarose, and a 10-μl aliquot was saved for input control. The rest of the sample was immunoprecipitated with 3 μg of the following antibodies overnight: Rabbit IgG (Cell Signaling), Histone H3 (Abcam), Histone H3 Acetyl K9 + K14 + K18 + K33 + K27 (Abcam). Antibody-nucleoprotein complexes were recovered by incubating 60 μl of salmon sperm DNA/protein A/G agarose for 1 h at 4 °C with rotation. Beads were washed and antibody-nucleoprotein complexes were eluted from protein A/G agarose beads in elution buffer de-cross-linked by adding 20 μl 0.5 M NaCl and incubated at 65 °C. Subsequently, RNAsae A and proteinase K were added and DNA fragments were purified using QIAquick PCR purification kit (Qiagen). Semi-quantitative PCR was performed with the following primers: Epas1 Forward: 5'-CATTACTC AGTCTGGCCTAAC-3'; Epas1 Reverse: 5'-CTCAGGACAGCTCCGATG TGTA-3'; Hif1a Forward: 5'-AATACCTTGAGAGCCTCTGTGT-3'; Hif1a Reverse: 5'-CAGTTGCCTTCAGCCATCGAGA-3'.

Oncomine study design. We used publicly available databases through Oncomine Research Premium edition software (version 4.5, Life Technologies) to query HIF-2α expression and survival data in sarcoma relevant to our study. For expression data, we evaluated the Barretina et al. (GEO accession number GSE21124) and Gobble et al. data sets (GEO accession number GSE39929)13,35, and for survival data the Gobble et al. data set was analysed.

Survival analysis. Available patient survival data were obtained from Gobble et al. data set for liposarcoma patients through Oncomine. Patients were divided into two groups: tumours expressing the highest 50% of EPAS1 'High EPAS1' and lowest 50% 'Low EPAS1', and Kaplan–Meier analyses were performed for overall survival of patients.

RNA-seq. RNA was extracted from KP and KPH2 FFPE tumour sections using RNeasy FFPE kit (Qiagen #73504). Library prep and RNA-seq were performed by the Next Generation Sequencing Core at the University of Pennsylvania using TruSeq Stranded Kit (Illumina #RS-122-2201), Ribo-Zero Gold Kit (Epicentre #MRZG126) and an HiSeq 100SR (Illumina). Raw sequence and quality files were also exported from DESeq2 for visualization and used as input for Gene Set Enrichment Analysis (see below). Overall sample relatedness was visualized with PCA (as implemented in Partek Genomics Suite v6.6, Partek, Inc.).

Differential expression and gene set enrichment analysis. Transcript count files for 3 HIF-2α knockdown samples and four wild-type samples were analysed with DESeq2 (version 1.6, http://bioconductor.org/packages/release/bioconductor/html/DESeq2.html) for differential expression. Log2-transformed, normalized intensities were also exported from DESeq2 for visualization and used as input for Gene Set Enrichment Analysis (see below). Overall sample relatedness was visualized with Principal Components Analysis (PCA, as implemented in Partek Genomics Suite v6.6, Partek, Inc. St. Louis, MO) using the normalized expression intensities for all transcripts. Log2-transformed expression intensities from these samples were also exported from DESeq2 for visualization and used as input for Gene Set Enrichment Analysis (see below).

TCGA bioinformatics analysis. Level 3 data comprising regions with altered copy number and methylation status in sarcoma were downloaded through the TCGA portal (https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp) on 17 December 2014. Data were analysed with Partek software (version 6.6). Tumour samples for copy number analysis consisted of 260 primary tumours, 3 recurrent tumours and 1 metastatic tumour. The results from blood and normal controls were eliminated. Portal (https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp) on 17 December 2014. Number and methylation status in sarcoma were downloaded through the TCGA Bioinformatics Analysis (FDR) index.jsp). Unique transcript alignments were counted for each sample using the normalize expression intensities for all transcripts. Log2-transformed expression intensities from these samples were also exported from DESeq2 for visualization and used as input for Gene Set Enrichment Analysis (see below). Overall sample relatedness was visualized with PCA (as implemented in Partek Genomics Suite v6.6, Partek, Inc.).

Ingenuity pathway analysis. DESeq2 statistical results for differential expression were uploaded to IPA. Genes with at least 0.75 log2 ratio (± 1.7 fold) and a FDR corrected p-value of <0.05 were analysed. The changes in the subset of these genes that were known to interact with MTOR were used to predict the activity changes of MTOR in KPH2 versus KP tumours. Canonical pathway analysis was also performed, with the top 15 most statistically significant pathways shown (orange bars, pathway predicted to be more active in KPH2 tumours; blue bars, pathway predicted to be less active in KPH2 tumours; grey bar, no activity pattern available).

Statistical analysis. Statistical analysis was performed using Prism (GraphPad Software). Data are shown as mean ± s.e.m. unless otherwise specified. Data were reported as biological replicates with technical replicates indicated in figure legends. Unpaired two-tailed Student t-tests were performed to determine if results were statistically significantly different, with a P-value cutoff <0.05 considered significant. Variation within each data group was measured with Graphpad Prism software, and found to be similar between groups tested.

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Author contributions
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Additional information
Accession codes: The RNA-seq data have been deposited in the Gene Expression Omnibus database under accession code: GSE67672.

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