学位論文

論文題目

The oncogene-dependent resistance to reprogramming
unveils cancer therapeutic targets

がん細胞の iPS 細胞化を阻害する
分子メカニズムの解明及び
その特性を応用した新しい薬剤スクリーニング系の開発

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Summary

The resistance to transcription factor-mediated reprograming into pluripotent stem cells is one of the distinctive features of cancer cells. Here, I dissect the profiles of the reprogramming factor binding and the subsequent transcriptional response in cancer cells to reveal the molecular mechanisms underlying this phenomenon. Using clear cell sarcomas (CCSs) as a model, I show that expression of the driver oncogene *EWS/ATF1* misdirects the reprogramming factors to cancer-specific enhancer elements and thereby impairs the early transcriptional response toward pluripotency which is otherwise provoked. Consistently, sensitization to the reprogramming cue is also observed in other types of cancer when the corresponding oncogenic signals are pharmacologically inhibited. Exploiting this oncogene dependence of the transcriptional 'stiffness', I identify the mTOR signaling pathway downstream of EWS/ATF1, and discover inhibiting the mTOR activity substantially attenuates the propagation of CCS cells both *in vitro* and *in vivo*. Collectively, my results demonstrate that the early transcriptional response to cell fate perturbations can be a faithful readout to identify effective therapeutics in cancer cells. Moreover, the current study has implications for understanding how the cancer cell identity is robustly maintained.

Introduction

Somatic cell identity is maintained through coordinated regulation of the cell type-specific transcriptional network and the epigenetic landscape, especially at distal enhancer elements (Bulger and Groudine, 2011; Hnisz et al., 2016; Spitz and Furlong, 2012; Whyte et al., 2013). However, somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) by the forced expression of reprogramming transcription factors: OCT4 (encoded by *Pou5f1*, also known as OCT3 and OCT3/4), SOX2, KLF4, and c-MYC (hereafter referred to as OSKM) (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). During the reprogramming process, global reorganization of both transcriptional and epigenetic regulation takes place (Polo et al., 2012). Particularly, the recruitment of OSKM to cell type-specific enhancers represses their activity and is the early critical event during OSKM-mediated reprogramming (Chronis et al., 2017; Shibata et al., 2018). Consistent with this, the expression of lineage-specific transcription factors (TFs), which maintain the cell type-specific transcriptional network, interferes with OSKM-induced reprogramming toward pluripotency both *in vitro* and *in vivo* (Hanna et al., 2008; Hikichi et al., 2013; Shibata *et al.*, 2018). Therefore, for successful reprogramming, the intrinsic somatic cell program must be shut down.

It is generally recognized that cancer cells are more resistant to reprogramming into PSCs. Nevertheless, several studies have succeeded in generating PSCs from cancer cells. For example, oncogenic *RAS*-induced murine melanoma nuclei and cells have been successfully reprogrammed into PSCs by the embryonic stem cell (ESC) derivation following nuclear transfer, and by induction of OSKM, respectively (Hochedlinger et al., 2004; Utikal et al., 2009). Similarly, iPSC-like cells have been derived from human cancer cells such as chronic myeloid leukemia (CML) cells (Carette et al., 2010; Kumano et al., 2012) and pancreatic ductal adenocarcinoma cells (Kim et al., 2013). Despite the successful derivation of iPSCs from cancer cells, the efficiency is considerably lower than somatic cell reprogramming (Kim and Zaret, 2015; Komura et al., 2019), which supports the notion that cancer cells are more resistant to reprogramming. However, the underlying mechanisms of cancer cell resistance to reprogramming remain unknown.

Clear cell sarcoma (CCS) is a rare soft tissue sarcoma and characterized by reciprocal

chromosomal translocations resulting in expression of the chimeric *EWS/ATF1* fusion gene. CCS is a particularly deadly type of sarcoma, as no effective therapeutics could be achieved. Previous studies have demonstrated that expression of the *EWS/ATF1* fusion gene is essential for the development and maintenance of CCSs *in vivo* (Straessler et al., 2013; Yamada et al., 2013), indicating that *EWS/ATF1* is a key driver oncogene in CCSs. A recent study showed that *EWS/ATF1*-induced mouse CCS cells are reprogrammable into iPSCs that can in-turn differentiate into adult somatic cells *in vivo* (Komura *et al.*, 2019). In the present study, using CCS as a model, I demonstrate that an oncogenic driver signal impairs the early transcriptional response to reprogramming factors in cancer cells. Mechanistically, expression of the key driver oncogene misdirects the transduced reprogramming TFs to cancer-specific enhancers. On the basis of the oncogene-dependent resistance to reprogramming, I show that a driver oncogene *EWS/ATF1* activates a mTOR signaling pathway in CCSs, and inhibition of the mTOR signaling remarkably inhibits the propagation of CCS cells. My results imply that an oncogenic driver signal restricts the cancer cell plasticity upon cell fate perturbations, which could be used to identify a promising therapeutic target in each cancer type.

Results

EWS/ATF1 **inhibits cellular reprogramming of murine CCS cells**

In the previous study, I succeeded in establishing iPSCs from murine CCS cells (the G1297 cell line) that harbor doxycycline (Dox)-controllable *EWS/ATF1* alleles (Komura *et al.*, 2019). In the present study it was found that, after *OSKM* transduction (human *OSKM*), iPSC-like colonies appeared from G1297 CCS cells lacking *EWS/ATF1* expression (No Dox), but not CCS cells expressing *EWS/ATF1* (treated with 0.2 μg/ml Dox) (Fig. 1A, 1B, S1A, S1B). Moreover, the number of iPSC-like colonies was inversely correlated with the expression levels of *EWS/ATF1* (Fig. 1C). These results imply that expression of the driver oncogene *EWS/ATF1* inhibits derivation of iPSCs from CCS cells.

To uncover the kinetics of the *EWS/ATF1*-mediated inhibition of CCS cell reprogramming, the presence of SSEA1 positive (+) cells was examined, which emerge in the early stage of OSKM reprogramming. The number of SSEA1+ cells in *OSKM*-induced CCS cells (*OSKM*-CCS cells) was markedly decreased by *EWS/ATF1* expression (Fig. 1D), indicating that *EWS/ATF1* abrogates the early stage of the reprogramming. Of note, the expression of *EWS/ATF1* did not reduce the number of SSEA1+ cells in *OSKM*-induced mouse embryonic fibroblasts (MEFs) (Fig. S1C), which suggests that the impaired reprogramming is specific to CCS cells.

EWS/ATF1 **expression impairs the early transcriptional response to reprogramming factors toward pluripotency in CCS cells**

Considering that *EWS/ATF1* inhibits the early reprogramming of CCS cells, the early transcriptional response to OSKM was examined (Fig. S1D). RNA-seq analysis revealed that a number of genes were upregulated only in the absence of *EWS/ATF1* expression in *OSKM*-CCS cells (Fig 1E, Cluster 1 in Fig. 1F, S1E－G). Notably, both *Fbxo15* and *Podxl*, early reprogramming markers of MEFs were included in the Cluster 1 (Fig. 1F). *Myb* and *Dmrtc2*, two TFs that are expressed in intermediate cells poised for reprogramming (Schwarz et al., 2018), were similarly increased in the absence of *EWS/ATF1* expression (Fig. 1E, 1F, S1F, S1G). In the presence of *EWS/ATF1* expression, the aforementioned genes showed the same pattern of

expression; however, the degree of gene promotion was often less compared with the *OSKM*-CCS cells without *EWS/ATF1* expression (Fig. 1E, 1F, S1F, S1G). Remarkably, Cluster 1 genes in *OSKM*-CCS cells often exhibited upregulation during early stage of MEF reprogramming (from SSEA+ day 3 to SSEA+ day 6) (Fig. 1G), implying that *EWS/ATF1* expression inhibits the early transcriptional response toward pluripotency. Consistently, RT-qPCR showed that expression of *Cdh1*, a marker of mesenchymal-to-epithelial transition (MET) that occurs at the initial stage of reprogramming (Li et al., 2010; Samavarchi-Tehrani et al., 2010), is increased in *OSKM*-CCS cells without *EWS/ATF1* expression (Fig. S1H). Moreover, expression of *Nanog*, an important TF for the pluripotency network, also increased, albeit at very low levels, in the absence of *EWS/ATF1* expression (Fig. 1H). There was a set of genes that were upregulated only in the presence of *EWS/ATF1* expression following OSKM transduction (Cluster 3 in Fig. 1F, S1G), indicating that *EWS/ATF1* expression induces the aberrant transcriptional response to OSKM.

I also examined the effect of *EWS/ATF1* expression on *MYOD1*-mediated muscle trans-differentiation in CCS cells (Fig. S2A) (Weintraub, 1993). Notably, *EWS/ATF1* expression inhibited the emergence of myosin heavy chain (MHC)+ cells in *MYOD1*-induced CCS cells (*MYOD1*-CCS cells) (Fig. 1I). The transcriptional response to exogenic MYOD1 was enhanced in a large number of genes in the absence of *EWS/ATF1* expression (Fig. 1J, Cluster 1 in Fig. 1K, S2B). Remarkably, Cluster 1 genes in *MYOD1*-CCS cells included *Myog* and were related to skeletal muscle differentiation (Nabeshima et al., 1993) (Fig. 1J–1L, S2B, S2C). Moreover, genes associated with MYOD1-binding in myoblasts were often more upregulated in the absence of *EWS/ATF1* expression in *MYOD1*-CCS cells (Fig. S2D). As observed in *OSKM*-CCS cells, a subset of genes was preferentially upregulated in the presence of *EWS/ATF1* expression (Cluster 3 in Fig. 1K). Collectively, I conclude that the driver oncogene *EWS/ATF1* impairs the early response to reprogramming factors in CCS cells.

Cancer-specific enhancer elements are a target of exogenous reprogramming TFs binding in CCS cells

I next tried to gain mechanistic insights into the distinct transcriptional response of CCS cells to reprogramming factors. ChIP-seq analysis of exogenous TFs was performed to examine the effect

of *EWS/ATF1* expression on the binding patterns of reprogramming TFs in *OSKM*-CCS cells and *MYOD1*-CCS cells (Fig. S3A, S3B). It was confirmed that *EWS/ATF1* expression does not reduce the levels of transgenic OCT4 protein in *OSKM*-CCS cells (Fig. S3C). Unexpectedly, the number of OCT4 and MYOD1 binding sites were larger in *EWS/ATF1*-expressing CCS cells, which display the impaired transcriptional response to each reprogramming TF (Fig. 2A). Motif analysis revealed that OCT4 and MYOD1 binding regions in *EWS/ATF1*-expressing CCS cells frequently harbor ATF/CRE-related motifs, a target of EWS/ATF1 binding, in addition to the OCT4 (*Pou5f1*) and MYOD motif, respectively (Fig. 2B). In addition, using all the reprogramming TF binding regions as a background set revealed that these EWS/ATF1 associated motifs were particularly enriched in the peaks unique to cells expressing EWS/ATF1 (Fig. S3D, S3E). This result strongly suggests that the reprogramming TFs were directed by EWS/ATF1 to generate the distinct binding patterns. Consistently, the regions with the enhanced TF binding in *EWS/ATF1*-ON cells were often bound by EWS/ATF1 in CCS cells (Fig. 2C, 2D) (Komura *et al.*, 2019). Furthermore, these regions exhibited a higher enrichment of H3K27ac in CCS cells (Fig. 2C, 2D) (Komura *et al.*, 2019). Given that EWS/ATF1 acts as a transcriptional activator and EWS/ATF1-binding regions are frequently overlapped with H3K27ac-marked regions (Komura *et al.*, 2019), these results collectively imply that the exogenous reprogramming TFs bind to active enhancer elements established by EWS/ATF1 in CCS cells. Consistent with this, reprogramming TF binding at cancer-specific enhancer elements was abrogated after withdrawal of *EWS/ATF1* (Fig. 2C, 2D, S3F). The same binding patterns were observed when another ChIP-seq data with an antibody for OCT4 were plotted to the same genomic coordinates, which further supports my conclusion (Fig. S3F).

All together, I propose that cancer-specific enhancer elements entrap exogenous reprogramming factors, which causes the impairment of transcriptional response toward pluripotency in cancer cells. This is consistent with previous findings that reprogramming TFs bind to enhancer elements during the initial stage of somatic cell reprogramming (Chronis *et al.*, 2017). Of note, I found that the OCT4 binding sites tended to reside in a closer distance to their nearby genes when the genes are upregulated upon the reprogramming TF expression (genes at Cluster 1 and 3 in *MYOD1*-CCS cells [Fig. 1K] and *OSKM*-CCS cells [Fig. 1F]) (Fig. S3G). This

result suggests that the aberrant binding of reprogramming TFs at EWS/ATF1-mediated enhancers is responsible for the distinct transcriptional response in CCS cells.

Inhibition of the oncogenic signal facilitates the transcriptional response to reprogramming factors in various cancer types

Based on the above findings, I next determined whether other cancer types also exhibit the oncogene-dependent resistance to the OSKM-mediated reprogramming. For this purpose, I introduced an *OSKM*-inducible allele (murine *OSKM*) in human cancer cell lines using a *piggyBac* transposon system (Fig. S4A) (Woltjen et al., 2009; Yagi et al., 2019) and investigated the early transcriptional response at 48 hours post-*OSKM* induction. I first examined the response in an *OSKM*-controllable lung cancer cell line HCC827 (*OSKM*-HCC827) containing an activating driver mutation at *EGFR* that is required for propagation (Weinstein and Joe, 2006) (Fig. S4B, S4C). Notably, RNA-seq analysis revealed that treatment with gefitinib, a specific kinase inhibitor against EGFR, typically increased the expression of genes that are commonly upregulated in partially reprogrammed human cells, including *DNMT3L*, *NLRP7*, and *APOL4* (Rand et al., 2018) (Fig. 3A, S4D). RT-qPCR analysis showed that expression of *NANOG* was also increased, albeit at low levels, by gefitinib treatment of *OSKM*-HCC827 cells (Fig. 3B). By sharp contrast, treatment with other kinase inhibitors, or a representative chemotherapeutic drug (5-fluorouracil, 5FU), did not increase *NANOG* expression (Fig. 3B). Moreover, treatment with siRNAs targeting *EGFR*, but not *FGFR*, significantly increased *NANOG* expression in *OSKM*-HCC827 cells (Fig. 3C, S4E).

Remarkably, the augmented expression of *NANOG* upon *OSKM* transduction was similarly observed in human *HER2*-amplifying breast cancer cells (SK-BR3), chronic myelogenous leukemia cells harboring the *BCR-ABL* fusion (K562), and *KRAS*-mutated lung cancer cells (A549) by inhibition of each cancer type-specific oncogenic signal (lapatinib, imatinib, and trametinib, respectively) (Fig. 3D, S4B, S4C). Although I observed increased *NANOG* expression in response to exogenous OSKM in multiple cancer types, the levels of *NANOG* expression were considerably lower than those of human PSCs (Fig. 3C). Further, *OSKM*-induced cancer cells could not be fully reprogrammed to obtain iPSCs, even after an extended period of culture (data

not shown). Taken together, I conclude that a driver oncogenic signal impairs the early transcriptional response to OSKM toward iPSCs in various types of cancer cells.

The transcriptional response to OSKM in patient-derived cancer cells reflects the clinical response to a cancer drug

I next examined the early transcriptional response to OSKM in patient-derived cells (PDCs) from a patient with a non-small cell lung cancer harboring a *EML4-ALK* fusion gene (LCC-028-3 cells) (Fig. S4B, S4C) (Sakamoto et al., 2011). *OSKM*-induced LCC-028-3 cells (*OSKM*-LCC-028-3 cells) exhibited increased *NANOG* expression after treatment with alectinib, a kinase inhibitor against ALK (Fig. 3E). This was consistent with the patient response to clinical alectinib treatment (Sakamoto *et al.*, 2011). Notably, the increase in *NANOG* expression by alectinib treatment was abrogated in *OSKM*-induced LCC-028-4 and LCC-028-5 cells, two PDCs from the same patient after acquisition of clinical resistance to alectinib (Fig. 3F) (Sakamoto *et al.*, 2011). These results demonstrate that the transcriptional response to OSKM in PDCs reflects the clinical response to the drug in the cancer patient.

Screen of signaling pathways that abrogate the early transcriptional response to OSKM toward pluripotency in cancer cells

I found that a cancer type-specific oncogenic signal impairs the transcriptional response to cell fate perturbations in various cancer types. Considering that a driver oncogenic signal remains to be uncovered for most cancer types, these findings prompted me to devise a screening system to determine an intracellular signaling pathway that causes the resistance to OSKM-mediated reprogramming, which could also be candidate pathways for a driver oncogenic signal. Given that *NANOG* is an early response gene during human reprogramming, as well as an authentic marker gene of pluripotency in human PSCs, I utilized its expression as a representative of the early transcriptional response to OSKM toward pluripotency. The screen of *NANOG* expression was performed by RT-qPCR in combination with a SCADS inhibitor kit that contains a library of 361 bioactive compounds that may regulate intracellular signaling pathways in *OSKM*-inducible cancer cells. Since certain chemical compounds may affect *OSKM* transgene expression, and

subsequently the reprogramming process (Huangfu et al., 2008), I also monitored the expression levels of exogenous reprogramming factors. Accordingly, the expression levels of *NANOG*, exogenic *mOct4* (*Pou5f1*), and *GAPDH* were simultaneously determined 2–3 days after treatment with Dox and test compounds using TaqMan probes (Fig. 4A).

To determine whether this screening system can identify a driver oncogenic signaling pathway, I first performed a pilot chemical screen using *OSKM*-HCC827 cells (n = 1; test compounds: 0.01 µM; Dox: 2 µg/ml; 48 hour treatment; 96-well plate). After the pilot test, compounds that exhibited a more than 2-fold increase in *NANOG* expression after normalization by both *GAPDH* and m*Oct4* (*NANOG*/*GAPDH* >2, *NANOG*/*mOct4* >2) were determined. Remarkably, out of 13 compounds that enhanced the *NANOG* expression, EGFR inhibitors, gefitinib and erlotinib, were included (Fig 4B, S5A).

I performed an additional pilot screen using an *OSKM*-inducible A549 cell line harboring a *KRAS* mutation (*OSKM*-A549 cells). A total of 980 chemical compounds (361 compounds in the SCADS inhibitor kit plus 619 inhibitors) was examined for an effect on *NANOG* expression in the *OSKM*-A549 cells (n = 1; test compounds: 1 µM; Dox: 2 µg/ml; 48 hour treatment; 96-well plate). Notably, selumetinib and binimetinib, inhibitors of MEK, which act downstream against *KRAS*, augmented *NANOG* expression (Fig. 4C, 4D, S5B). Furthermore, the vast majority of compounds that enhanced *NANOG* expression (*NANOG*/*GAPDH* >2, *NANOG*/*mOct4* >2) were inhibitors of KRAS-related signaling pathways (Fig. 4D). Some HDAC inhibitors, such as trichostatin A and CUDC-907, increased *NANOG* expression when normalized by *GAPDH* (Fig. 4C). However, these inhibitors also increased transgenic *mOct4* expression, which abrogated *NANOG* upregulation after normalization by *mOct4* expression (Fig. S5B).

Additionally, the *OSKM*-LCC-028-3 PDCs harboring a *EML4-ALK* fusion gene were screened with the SCADS inhibitor kit (361 compounds) and alectinib as a positive control (n = 2; test compounds: 1 µM; Dox: 0.5 µg/ml; 72 hour treatment; 96-well plate). The ALK inhibitors crizotinib and alectinib were among the 12 compounds that enhanced *NANOG* expression (*NANOG*/*GAPDH* >4, *NANOG*/*mOct4* >4) (Fig. 4E, S5C). Most of the compounds that inhibited cell growth did not increase *NANOG* expression (Fig. 4F, S6A–C), demonstrating that *NANOG* upregulation was indirectly associated with cell growth *in vitro*. Moreover, this observation

supports the idea that exploiting the resistance to the OSKM-mediated reprogramming can enrich compounds targeting driver oncogenic signals more efficiently compared to examining cell proliferation or survival.

Effective combination of molecularly targeted drugs further augments the early transcriptional response to OSKM

The screen of the *KRAS*-mutated A549 cells revealed that ponatinib, a receptor tyrosine kinase (RTK) inhibitor, enhanced *NANOG* expression (Fig. 4D). Previous studies demonstrated that ponatinib, in combination with a MEK inhibitor, trametinib, synergistically inhibited cell propagation of *KRAS*-mutated cancer cells both *in vitro* and *in vivo* (Kitai et al., 2016; Manchado et al., 2016). Remarkably, the combinatorial treatment of ponatinib and trametinib had an additive effect on *NANOG* expression in both the *OSKM*-induced lung (A549) and pancreatic (PANC1) cancer cell lines harboring a *KRAS* mutation (n = 3; Dox: 2 μg/ml; 48 hour treatment; 6-well plate) (Fig. 4G). These results suggest that the transcriptional response to exogenous OSKM could be used to identify a combination of drugs for effective clinical responses.

Uncovering mTOR signaling as a driver oncogenic pathway in CCSs

I next tried to identify a driver oncogenic pathway in CCSs. Although the *EWS/ATF1* fusion gene is a driver oncogene in CCS (Straessler *et al.*, 2013; Yamada *et al.*, 2013), the downstream effector pathways of EWS/ATF1 remain unknown. Therefore, I applied the compound screen to human CCS cell lines MP-CCS-SY and KAS, which harbor the type1 and type2 *EWS/ATF1* fusion gene, respectively (Fig. S7A). I first confirmed that *EWS/ATF1* knockdown, using siRNAs targeting the breakpoint of the fusion gene, augments *NANOG* expression in the human *OSKM*-induced CCS cell lines (*OSKM*-MP-CCS-SY and *OSKM*-KAS) (Fig. 5A, S7B). Then, chemical compounds in the SCADS inhibitor kit (361 compounds) were screened for *NANOG* expression in the *OSKM*-induced CCS cell lines (n = 2 for each cell line; test compounds: 1 μM; Dox: 0.5 μg/ml; 48 hour treatment; 96-well plate).

I found that 67 and 33 inhibitors increased the *NANOG* expression in the *OSKM*-MP-CCS-SY and *OSKM*-KAS cells, respectively (*NANOG*/*GAPDH* >2, *NANOG*/*mOct4* >2) (Fig. 5B, S7C,

S7D). Of the 18 compounds that augmented *NANOG* expression in both cell lines (Fig. 5C), I focused on the mTOR inhibitors because all mTOR inhibitors from the SCADS kit (n=4) were included in the list. Notably, the mTOR inhibitors suppressed CCS cell growth/survival in a concentration-dependent manner *in vitro* (Fig. 5D). Moreover, siRNA treatment targeting *MTOR* (the catalytic subunit of the mTOR complex), as well as *RPTOR* and *RICTOR* (the major components of mTORC1 and mTORC2, respectively) (Laplante and Sabatini, 2012), all reduced CCS cell growth (Fig. 5E, S8A). Remarkably, knockdown of the type1 *EWS/ATF1* fusion gene in MP-CCS-SY cells resulted in decreased phosphorylation of the mTOR targets, including S6RP, 4EBP1, and AKT (Fig. 5F, S8B), demonstrating that *EWS/ATF1* activates the mTOR signaling pathway in CCS cells. The decreased S6RP phosphorylation was similarly observed in KAS cells after knockdown of the type2 *EWS/ATF1* fusion gene (Fig. S8B). Consistent with this, *EWS/ATF1* knockdown in MP-CCS-SY cells suppressed target genes of mTORC1 signaling (Fig. S8C). Indeed, affected genes by *EWS/ATF1* knockdown were similarly altered after knockdown of *MTOR* in MP-CCS-SY cells (Fig. S8D, S8E). Moreover, induction of mTOR activation using a gain-of-function mutant of mTOR (Ohne et al., 2008) partially rescued the suppressive effect on cell growth by *EWS/ATF1* knockdown (Fig. S8F−H). Finally, I tested whether a mTOR inhibitor suppresses *in vivo* propagation of CCS cells. Rapamycin substantially attenuated the growth of both MP-CCS-SY and KAS cells in xenograft models (Fig. 5G, S8I). Taken together, the mTOR signaling pathway is a driver oncogenic pathway activated by EWS/ATF1, thus it offers a therapeutic target for CCSs.

Effective combinatorial treatment for mTOR inhibitors in CCSs

To explore the most effective combination of mTOR inhibitors for CCSs, I next aimed to identify the compounds that further augment the transcriptional response to OSKM in combination with rapamycin. I first tested, in combination, 14 compounds that enhanced *NANOG* expression in the *OSKM*-CCS cell lines and were distinct from mTOR inhibitors (Fig. 5C) (rapamycin: 5 nM; test compounds: lower concentration of either IC_{50} or 1 μM; 48 hour treatment). However, I did not observe a remarkable increase in *NANOG* expression by the combinatorial treatment (Fig. S9A). Accordingly, I expanded the test compounds for the combination assay. Fifteen compounds that

augmented *NANOG* expression in either of the *OSKM*-CCS cell lines were examined for additional effects on the *NANOG* expression in rapamycin-treated *OSKM*-CCS cell lines (rapamycin: 5 nM; test compounds: 10 μM; 48 hour treatment). I found that PD16316, a p38 inhibitor, enhanced *NANOG* expression in combination with rapamycin in the *OSKM*-CCS cell lines, which was especially prominent in *OSKM*-MP-CCS-SY cells (Fig. 6A, 6B, S9B−D). Consistent with this, treatment with siRNAs targeting *MAPK14* that encodes p38alfa, a major isoform of p38, similarly increased *NANOG* expression in rapamycin-treated *OSKM*-CCS cell lines (Fig. 6C, S9E, S9F). This affirmed that the combinatorial inhibition of mTOR signaling and the p38 pathway augments the early transcriptional response to OSKM toward pluripotency.

Finally, I tested whether the combinatorial treatment of p38 inhibitors with rapamycin was effective in suppressing CCS propagation. A single treatment of a p38 inhibitor had a suppressive effect on CCS cell growth to variable degrees *in vitro* (Fig. S9G). Similarly, *MAPK14* knockdown caused a modest reduction in cell proliferation/survival in CCS cells (Fig. S9H). Notably, the combinatorial treatment of p38 inhibitors with rapamycin showed an augmented inhibition in cell proliferation of MP-CCS-SY cells *in vitro* (Fig. 6D). The additive effect was similarly observed in CCS cells treated with rapamycin and siRNAs targeting *MAPK14* (Fig. S9H). I further accessed the inhibitory effect of the combinatorial treatment on *in vivo* propagation in the xenograft model. Remarkably, BIRB796 treatment enhanced anti-tumor effects of rapamycin in CCS cells *in vivo* (Fig. 6E, S10A-C).

Discussion

It is recognized that cancer cells are more resistant to *OSKM*-mediated reprogramming into iPSCs, suggesting that their transcriptional network and epigenetic regulation are stably maintained upon cell fate perturbations. Here, I found that exogenous reprogramming TFs preferentially bind to cancer-specific enhancer elements in CCS cells. Previous studies have demonstrated that reprogramming TFs are recruited to somatic cell enhancer elements, which represses the transcriptional program in the initial stage of somatic cell reprogramming (Chronis *et al.*, 2017). Given that cancer cells exhibit distinctive enhancer landscapes (Dunham et al., 2012; Fulco et al., 2016; Kundaje et al., 2015; Schuijers et al., 2018; Thurman et al., 2012), my results suggest that failed repression of a cancer-specific transcriptional program sustains aberrant binding of OSKM at cancer-specific enhancer elements. This eventually causes an impairment in the early transcriptional response to OSKM toward pluripotency in cancer cells. Consistent with this, withdrawal of the driver oncogene in CCS cells abrogated OCT4 binding at cancer-specific enhancer elements and augmented the transcriptional response to OSKM toward pluripotency. These results may have important implications for understanding how cancer cell identity is maintained.

Given that genetic aberrations drive cancer development and survival, downstream signaling pathways have offered a promising target for cancer therapeutics. Despite a large number of genetic mutations, cancer cell survival depends on relatively few mutations and signaling pathways: a concept referred to as "oncogene addiction" (Pagliarini et al., 2015; Weinstein, 2002). Indeed, this concept has supported a cancer therapy strategy of molecularly targeting and inhibiting a specific oncogenic pathway (Sharma and Settleman, 2007). However, a specific effector pathway that drives cancer development and survival remains undetermined for many cancer types. In this study, I showed that a representative molecularly targeted drug against a key driver oncogenic signal exclusively augments the early transcriptional response to OSKM in different cancer types. Furthermore, exploiting this oncogene-dependent resistance to cancer cell reprogramming, a mTOR pathway was identified as a key effector signal in CCSs, which is activated by the oncogenic EWS/ATF1. Considering that resistance to reprogramming

is a general feature of cancer cells, I propose that this strategy may be applicable to a diverse range of cancer types in which the driver oncogenic signal remains to be uncovered.

Use of a combination of molecularly targeted drugs has exhibited remarkable clinical effects against cancer cell propagation (Al-Lazikani et al., 2012) that may be a viable therapeutic strategy. My compound screen revealed that treatment with trametinib and ponatinib, a potent therapeutic combination for *KRAS*-mutated cancers, augments the early transcriptional response to OSKM in *KRAS*-mutated cancer cells in an additive manner. Of note, a combinatorial treatment of a mTOR inhibitor with p38 inhibitors similarly augmented the transcriptional response in CCS cells and substantially inhibited CCS propagation *in vivo*. Successful identification of the effective combination of cancer drugs underscores a unique feature of my strategy. I also showed, using PDCs, that the transcriptional response to OSKM reflects the patient-specific clinical responsiveness to a molecularly targeted drug. Given that individual variability in the genome and environment has an impact on the biological behavior of cancer cells, personalized medicine holds great promise as a tailored treatment to ensure better patient care (Hamburg and Collins, 2010). My creening strategy, in combination with PDCs, may offer a novel platform to target a driver oncogenic signal and tailor the molecular therapy to a patient that can be modified over the clinical course.

In summary, taking advantage of reprogramming technologies, I demonstrate that a driver oncogenic signal induces the impaired transcriptional response to cell fate perturbations. My results may have implications for understanding how cancer cell identity is robustly maintained, which could be used to unveil promising targets for effective therapeutics across diverse cancer types.

Figure legends

Fig. 1. *EWS/ATF1* alters the early transcriptional response and inhibits reprogramming of CCS cells.

A. An experimental protocol for cancer cell reprogramming. *OSKM* were retrovirally introduced into *EWS/ATF1*-expressing G1297 CCS cells.

B. Brightfield images after OSKM transduction in G1297 CCS cells. No iPSC-like colonies were observed in *OSKM*-CCS cells expressing *EWS/ATF1*, whereas iPSC-like colonies emerged after the withdrawal of Dox. Scale bars: 300 μm.

C. The number of iPSC-like colonies (Day 19). The number was inversely correlated with expression levels of *EWS/ATF1*. The data are presented as mean ± SD of biological triplicates. ***P<0.001; one-way ANOVA, followed by the Dunnett's *post-hoc* test.

D. Flow cytometry analysis of SSEA1 expression in *OSKM*-CCS cells (Day 10). The number of SSEA1-positive cells was decreased by *EWS/ATF1* expression.

E. RNA-seq analysis showing transcriptional response to OSKM toward pluripotency. The response was impaired by *EWS/ATF1* expression. Differentially expressed genes (DEGs) (fold change >2, FDR<0.05) are labeled with pink in a volcano plot.

F. Heat map of RNA-seq expression z-scores. Expression profiles of genes upregulated (fold change >2) after the OSKM transduction were subjected to K-means clustering. Cluster 1 genes exhibit increased expression predominantly in the absence of *EWS/ATF1* expression, while Cluster 3 genes do so in the presence of *EWS/ATF1* expression. Cluster 2 genes show the increased expression in the absence of *EWS/ATF1* expression, but the induction levels are far less when compared with the effects of *EWS/ATF1* expression.

G. Gene set enrichment analysis (GSEA) of Cluster 1 genes in Fig. 1F for a gene set which are upregulated during the early stage of cellular reprogramming in MEFs (from SSEA+ day 3 to SSEA+ day 6 (Schwarz *et al.*, 2018)) (normalized enrichment score [NES] = 1.76).

H. RT-qPCR analysis for *Nanog* expression. Mean values of biological triplicates are shown. The expression level of *EWS/ATF1*-expressing *OSKM*-CCS cells was set to 1. **P<0.01, *P<0.05; unpaired two-tailed t-test.

I. Left: myosin heavy chain (MHC)-positive cells in *MYOD1*-CCS cells. Bars: 20 μm. Right: frequency of MHC-positive cells. The data are presented as mean ± SD of biological triplicates. *P<0.05; unpaired two-tailed t-test.

J. RNA-seq analysis showing transcriptional response to MYOD1. DEGs (fold change >2, FDR<0.05) are labeled with pink in a volcano plot.

K. Heat map of RNA-seq expression z-scores. Expression profiles of genes upregulated (fold change >2) after the MYOD1 transduction were subjected to K-means clustering. Cluster 1 genes exhibit increased expression after the MYOD1 transduction only in the absence *EWS/ATF1* expression, while Cluster 3 genes show the increased expression in the presence of *EWS/ATF1* expression. Cluster 2 genes show the increased expression regardless of *EWS/ATF1* expression.

L. Gene ontology (GO) terms associated with each cluster in Fig. 1K. GO terms and the p-values are shown.

Fig. 2. Cancer-specific enhancers are a target of exogenous OCT4 binding in cancer cells.

A. Venn diagrams showing the overlap between OCT4-binding peaks (upper) and MYOD1-binding peaks (lower) in *OSKM*-CCS cells and *MYOD1*-CCS cells, respectively. An antibody against the V5-tag (Abcam, ab15828) was used in the ChIP-seq analyses as the exogenous OCT4 and MYOD1 were V5-tagged.

B. De novo motif enrichment analysis for OCT4-binding peaks (upper) and MYOD1-binding peaks (lower) in *OSKM*-CCS cells and *MYOD1*-CCS cells, respectively. ATF/CREB-related motifs, in addition to the binding motifs of the reprogramming factors, were significantly overrepresented in the peaks.

C. Heatmap of ChIP-seq signals at OCT4-binding (upper) and MYOD1-binding (lower) peaks detected in at least either Dox-ON or Dox-OFF CCS cells. The binding peaks are clustered by the groups in A. EWS/ATF1 binding and H3K27ac enrichment are shown at the exogenous OCT4-binding sites (upper) and the MYOD1-binding sites (lower) in CCS cells. An antibody against the V5-tag was used.

D. Representative loci demonstrating binding of the reprogramming TFs at EWS/ATF1-associated enhancer elements in *OSKM*-CCS cells and *MYOD1*-CCS cells. *Fos* is one of the targets of EWS/ATF1 in G1297 CCS cells (Yamada *et al.*, 2013).

Fig. 3. The inhibition of driver oncogene signals facilitates the early transcriptional response to OSKM.

A. Upper panels: RNA-seq analysis showing transcriptional response to OSKM in *EGFR*-mutated HCC827 cells (gefitinib: IC50; Dox: 2 µg/ml; 48 hour treatment; 12-well plate). The transcriptional response to OSKM was augmented by gefitinib treatment. DEGs (fold change >2, FDR<0.05) are labeled with pink. Lower panel: Heat map of z-scores of commonly upregulated genes in partially reprogrammed human cells (TRA1-60 [+] cells on day 7) (Rand *et al.*, 2018). Low-expression genes (TPM < 3 in all samples) are excluded from the list.

B. RT-qPCR for *NANOG* expression in *OSKM*-HCC827 cells treated with various anti-cancer drugs (test compounds: IC50; Dox: 2 µg/ml; 48 hour treatment; 12-well plate). Data are presented as means ± SD of biological triplicates. The mean expression level of *NANOG* in DMSO-treated cells was set to 1. ***P<0.001; one-way ANOVA, followed by the Dunnett's *post-hoc* test.

C. RT-qPCR for *NANOG* expression in *OSKM*-HCC827 cells treated with siRNAs targeting *EGFR* or *FGFR* (Dox: 2 µg/ml; 48 hour treatment; 6-well plate). Data are presented as means ± SD of biological triplicates. The expression level of *NANOG* in human iPSCs was set to 1. ***P<0.001, *P<0.05; one-way ANOVA, followed by the Dunnett's *post-hoc* test.

D. RT-qPCR for *NANOG* expression in *OSKM*-SK-BR3, *OSKM*-K562, and *OSKM*-A549 cells treated with various anti-cancer drugs (test compounds: lower concentration of either IC50 or 10 µM; Dox: 2 µg/ml for *OSKM*-SK-BR3 and *OSKM*-A549 cells and 0.5 µg/ml for *OSKM*-K562 cells; 48 hour treatment; 12-well plate). The molecularly targeted drug for the corresponding cancer cells is shown in blue bars. Data are presented as means ± SD of biological triplicates. The mean expression level of *NANOG* in DMSO-treated cells was set to 1. ***P<0.001; one-way ANOVA, followed by the Dunnett's *post-hoc* test.

E. RT-qPCR for *NANOG* expression in *OSKM*-PDCs (LCC028-3 cells) treated with various anti-cancer drugs (test compounds: lower concentration of either IC50 or 10 µM; Dox: 0.5

µg/ml; 48 hour treatment; 12-well plate). LCC028-3 cells harbor the *EML4-ALK* fusion gene and thus are sensitive to alectinib. Data are presented as means ± SD of biological triplicates. The mean expression level of *NANOG* in DMSO-treated cells was set to 1. ***P<0.001; one-way ANOVA, followed by the Dunnett's *post-hoc* test.

F. RT-qPCR for *NANOG* expression in *OSKM*-PDCs (LCC028-3, -4, and -5 cells) treated with alectinib (alectinib: 1 or 0.1 µM; Dox: 0.5 µg/ml; 48 hour treatment; 6-well plate). LCC028-4 and -5 cells were derived from the same patient but after acquisition of alectinib resistance. Data are presented as means ± SD of biological triplicates. The mean expression level of *NANOG* in DMSO-treated cells in each PDCs was set to 1. **P<0.01, *P<0.05; one-way ANOVA, followed by the Dunnett's *post-hoc* test.

Fig. 4. Screen of signaling pathways that abrogate the early transcriptional response to reprogramming factors.

A. A schematic representation of the screening procedure.

B. A pilot screen of *OSKM*-HCC827 with 361 compounds in the SCADS inhibitor kit. Expression levels of *NANOG* (normalized to *GAPDH*) are shown. The mean expression level of *NANOG* in the control cells (DMSO) was set to 1. Note that erlotinib and gefitinib, both are EGFR inhibitors, augmented the *NANOG* expression.

C. A pilot screen of *OSKM*-A549 with 980 chemical compounds (361 compounds in the SCADS inhibitor kit plus 619 kinase inhibitors). Expression levels of *NANOG* (normalized to *GAPDH*) are shown. The mean expression level of *NANOG* in the control cells (DMSO) was set to 1. Note that MEK inhibitors, selmetinib and binimetinib augmented the *NANOG* expression. Trichostatin A and CUDC-907 are HDAC inhibitors.

D. Venn diagrams of a representative list of 24 compounds that exhibit more than 2-fold increase in *NANOG* expression after normalization by both *GAPDH* and m*Oct4* (*NANOG*/m*Oct4*>2, *NANOG*/*GAPDH*>2) in *OSKM*-A549 cells. Note that the majority of the compounds were related to the *KRAS*-signaling pathways.

E. A pilot screen of *OSKM*-PDCs (LCC-028-3) with 361 compounds in the SCADS inhibitor kit and alectinib. Average values of relative *NANOG* expression levels (normalized to *GAPDH*) of

two independent experiments are shown. The mean expression level of *NANOG* in the control cells (DMSO) was set to 1 in each experiment. Note that the ALK inhibitors, crizotinib and alectinib, augmented *NANOG* expression.

F. Scatter plots illustrating the correlation of *NANOG* expression levels (*NANOG/mOct4*) in *OSKM*-LCC-028-3 cells and the effect on cell number in LCC-028-3 cells. Note that most compounds that reduced cell number did not augment *NANOG* expression.

G. The combinatorial treatment with trametinib and ponatinib shows an augmentation in *NANOG* expression in *OSKM*-A549 and *OSKM*-PANC1 harboring a *KRAS* mutation (Dox: 2 µg/ml; 48 hour treatment; 6-well plate). Data are presented as the mean of three independent experiments. The mean expression level of *NANOG* in DMSO-treated cells in each experiment was set to 1.

Fig. 5. mTOR signaling is a driver oncogenic pathway in clear cell sarcomas.

A. RT-qPCR analysis for *NANOG* expression in *OSKM*-induced MP-CCS-SY and KAS cells treated with siRNAs targeting the type1 and type2 *EWS/ATF1* fusion genes, respectively (Dox: 0.5 µg/ml; 96 hour treatment for MP-CCS-SY and 48 hour treatment for KAS; 6-well plate). Data are presented as means ± SD of biological triplicates. The expression levels of *NANOG* in si*EWS/ATF1*-treated *OSKM*-induced cells were set to 1. ***P<0.001; one-way ANOVA, followed by the Dunnett's *post-hoc* test.

B. A screen of *OSKM*-MP-CCS-SY (top) and *OSKM*-KAS (bottom) cells with 361 compounds in the SCADS inhibitor kit. Average values of *NANOG* expression (normalized to *GAPDH*) of two independent experiments are shown. The mean expression level of *NANOG* in the control cells (DMSO) was set to 1 in each experiment.

C. Venn diagrams and a representative list of 18 compounds that augmented *NANOG* expression in both *OSKM*-MP-CCS-SY and *OSKM*-KAS cells.

D. Sensitivity of MP-CCS-SY and KAS cells to mTOR inhibitors. A cell-counting kit8 assay was performed after the drug treatment for 2 days.

E. Clonogenic assay of MP-CCS-SY and KAS cells treated with siRNAs against mTOR component genes for 72 hours (MP-CCS-SY) and 144 hours (KAS).

F. Western blot analysis revealed that the downstream effector of mTORC1 and mTORC2 were downregulated by knockdown of *EWS/ATF1* in MP-CCS-SY.

G. Relative tumor volumes of mice bearing MP-CCS-SY (up) and KAS (bottom) xenografts treated with DMSO and rapamycin (5 mg/kg body weight). Data are presented as means $\pm$ SD. *P<0.05; unpaired two-tailed t-test.

Fig. 6. Identification of an effective combinatorial treatment with mTOR inhibitors in clear cell sarcomas.

A. RT-qPCR for *NANOG* expression in *OSKM*-MP-CCS-SY cells after the combinatorial treatment with rapamycin (test compounds: 10 μM; rapamycin: 5 nM; Dox: 0.5 μg/ml; 48 hour treatment; 6-well plate). Data are presented as means $\pm$ SD of three independent experiments. The expression level of *NANOG* was normalized to *GAPDH* (left) and *mOct4* (right). The mean value of *NANOG* in DMSO-treated cells was set to 1 in each experiment. ***P<0.001, *P<0.01; one-way ANOVA, followed by the Dunnett's *post-hoc* test.

B. The combinatorial treatment with rapamycin and p38 inhibitors augmented *NANOG* expression in *OSKM*-MP-CCS-SY cells in an additive manner (Dox: 0.5 μg/ml; 48 hour treatment; 6-well plate). Data are presented as the mean of three independent experiments. The expression level of DMSO-treated cells in each experiment was set to 1.

C. RT-qPCR for *NANOG* expression in *OSKM*-MP-CCS-SY cells after treatment with siRNA targeting *MAPK14* (p38α) (Dox: 0.5 μg/ml; 48 hour treatment; 6-well plate). The combinatorial treatment with si*MAPK14* and rapamycin augmented *NANOG* expression in an additive manner. Data are presented as the mean of three independent experiments. The expression level of *NANOG* in DMSO/*siControl*-treated cells in each experiment was set to 1.

D. Clonogenic assay of MP-CCS-SY cells treated with rapamycin, p38 inhibitors, or a combination, as indicated.

E. Relative tumor volumes of mice bearing MP-CCS-SY xenografts treated with DMSO, rapamycin (2.5 mg/kg body weight), BIRB796 (50 mg/kg body weight), or a combination of both, for the indicated time points. Data are presented as means ± SD. ***P<0.001, **P<0.01, *P<0.05; unpaired two-tailed t-test.

Supplementary figure legends

Fig. S1. *EWS/ATF1* alters the early transcriptional response to reprogramming factors in CCS cells.

A. RT-qPCR for *EWS/ATF1* in G1297 cells. Data are presented as means ± SD of biological triplicates. The mean expression level of G1297 cells treated with 0.2 µg/ml Dox was set to 1. ***P<0.001, **P<0.01; one-way ANOVA, followed by the Dunnett's post-hoc test.

B. Effect of *EWS/ATF1* expression on the ESC growth. Dox concentration at 0.2 µg/ml had no effect on the ESC growth. ESCs containing Dox-controllable *EWS/ATF1* alleles at identical loci as G1297 CCS cells (Komura *et al.*, 2019) were used.

C. Flow cytometry analysis for SSEA1+ cells in *OSKM*-MEFs. *OSKM* were retrovirally introduced into *EWS/ATF1*-controllable MEFs. *EWS/ATF1* expression did not reduce the number of SSEA1+ cells in MEFs. MEFs containing Dox-controllable *EWS/ATF1* alleles at identical loci as G1297 CCS cells (Komura *et al.*, 2019) were used.

D. Schematic of the experimental protocol for RNA extraction and protein extraction. *OSKM* or *GFP* were retrovirally introduced into *EWS/ATF1*-expressing CCS cells (G1297).

E. RNA-seq analysis showing the comparable levels of transgene expression regardless of *EWS/ATF1* expression.

F. Expression of early reprogramming-related genes in RNA-seq analysis. *Fbxo15* and *Podxl* are early reprogramming marker genes. *Myb* and *Dmrtc2* are upregulated in intermediate cells poised to reprogramming from MEFs.

G. GO terms associated with each cluster in Fig. 1F. GO terms and the p-values are shown.

H. RT-qPCR for *Cdh1*. Data are presented as means ± SD of biological triplicates. The mean expression level of *EWS/ATF1*-expressing *OSKM*-CCS cells was set to 1. *P<0.05; unpaired two-tailed t-test.

Fig. S2. *EWS/ATF1* alters the early transcriptional response to MYOD1 in CCS cells.

A. An experimental protocol for *MYOD1*-induced myogenic differentiation.

B. Expression of representative genes related to skeletal muscle differentiation in RNA-seq analysis is shown.

C. RT-qPCR analysis for *Myog* expression. The data are presented as mean ± SD of biological triplicates. The expression level of *Myog* in *MYOD1*-CCS cells, in the absence of *EWS/ATF1* expression, was set to 1. ***P<0.001; unpaired two-tailed t-test.

D. RNA-seq analysis for MYOD1-binding associated genes in C2C12 (the nearest gene from the peak (Cao et al., 2010)), which exhibit increased expression levels in differentiated C2C12 cells (fold change >4 compared with CCS cells (Komura *et al.*, 2019; Marzi et al., 2012)). DEGs (fold change >2, FDR<0.05) in *MYOD1*-CCS cells are labeled with pink.

Fig. S3. Cancer-specific enhancers are a target of exogenous reprogramming TFs binding in cancer cells.

A. An experimental protocol for ChIP analysis in *OSKM*-CCS cells. V5-tagged human OCT4 together with human SKM was transduced in *EWS/ATF1*-inducible CCS cells. The V5-tag was fused in the N-terminus of human OCT4.

B. An experimental protocol for ChIP analysis in *MYOD1*-CCS cells. V5-tagged human MYOD1 was transduced in *EWS/ATF1*-inducible CCS cells. The V5-tag was fused in the C-terminus of human MYOD1.

C. Expression of exogenous OCT4 protein in G1297 CCS cells. The expression level of transduced OCT4 is not decreased in Dox-treated *OSKM*-CCS cells (0.2 µg /ml) that exhibit an impaired transcriptional response toward iPSCs.

D. A motif enrichment analysis for OCT4-binding peaks in *OSKM*-CCS cells using all the OCT4 binding peaks detected in the ChIP-seq analysis as a background.

E. A motif enrichment analysis for MYOD1-binding peaks in *MYOD1*-CCS cells using all the MYOD1 binding peaks detected in the ChIP-seq analysis as a background.

F. Heatmap of ChIP-seq signals with an OCT4 antibody at the OCT4 binding peaks detected in Fig. 2C. EWS/ATF1 bindings and H3K27ac enrichments are shown at the OCT4-binding sites in CCS cells.

G. Proximity of reprogramming TF binding peaks to TSSs of their nearby genes. The peaks are selected when their nearby genes belong to Cluster 1 or Cluster 3 (Fig. 1F: *OSKM*-CCS cells; Fig. 1K: *MYOD1*-CCS cells). Fractions of peaks with the indicated distance are shown.

Fig. S4. Inhibition of driver oncogenic signals facilitates the early transcriptional response to the OSKM reprogramming factors in human cancer cells.

A. A Dox-controllable *piggyBac* vector (*PB-OSKM* all-in-one) (Yagi *et al.*, 2019).

B. Derivation of *OSKM*-inducible human cancer cell lines and PDCs. The expression of *OSKM* after Dox treatment was confirmed by mCherry fluorescence and RT-qPCR in HCC827, SK-BR3, A549, PANC1, and LCC-028-3 PDCs. Scale bars: 300 µm. Data are presented as the mean ± SD of biological triplicates in RT-qPCR analysis. The mean expression level of *OSKM* in Dox-treated *OSKM*-HCC827 cells (2.0 µg/ml) was set to 1.

C. A schematic drawing of the experimental protocol for RNA extraction in *OSKM*-inducible human cancer cells.

D. Transcriptional response to OSKM in *OSKM*-HCC827 cells. The transcriptional response to OSKM was augmented by gefitinib treatment. *DNMT3L*, *APOL4*, and *NLRP7* are commonly upregulated genes in partially reprogrammed cells (Rand *et al.*, 2018).

E. Left: Knockdown efficiency for *EGFR* (left) and *FGFR* (right) in *OSKM*-HCC827 cells. Data are presented as the mean ± SD of biological triplicates in RT-qPCR analysis. The mean expression levels of *siControl*-treated cells were set to 1. Right: Western blot analysis for EGFR following siRNA treatment.

Fig. S5. Screen of signaling pathways that abrogate the early transcriptional response to reprogramming factors.

A. Left: a pilot screen of *OSKM*-HCC827 with 361 compounds in the SCADS inhibitor kit. Expression levels of *NANOG* expression (normalized to *mOct4*) are shown. The expression level of blank (DMSO) was set to 1. Right: Venn diagrams and a list of 13 compounds that exhibit more than 2-fold increase in *NANOG* expression after normalization by both *GAPDH* and m*Oct4* (*NANOG*/*mOct4*>2, *NANOG*/*GAPDH*>2). Note that EGFR inhibitors, erlotinib and gefitinib are included in the list.

B. A pilot screen of *OSKM*-A549 with 980 chemical compounds (361 compounds in the SCADS inhibitor kit plus 619 kinase inhibitors). Expression levels of *NANOG* expression (normalized to

mOct4) are shown. The expression level of *NANOG* in the control sample (DMSO) was set to 1. Trichostatin A and CUDC-907 are HDAC inhibitors.

C. Left: a pilot screen of *OSKM*-PDCs (LCC-028-3) with 361 compounds in the SCADS inhibitor kit and alectinib. Average values of relative *NANOG* expression levels (normalized to *mOct4*) of two independent experiments are shown. The expression level of *NANOG* in the control sample (DMSO) was set to 1 in each experiment. Right: Venn diagrams and a representative list of 12 compounds that exhibit more than 2-fold increase in *NANOG* expression after normalization by both *GAPDH* and m*Oct4* (*NANOG/mOct4*>2, *NANOG/GAPDH*>2). Note that ALK inhibitors, crizotinib and alectinib, are included in the list.

Fig. S6. The correlation of *OSKM*-induced *NANOG* expression and cell growth following treatment with chemical compounds.

A. Scatter plots illustrating the correlation of *NANOG* expression levels in *OSKM*-LCC-028-3 cells and the effect on cell numbers in LCC-028-3 cells, following treatment with test compounds. Average values of relative *NANOG* expression levels (normalized to *GAPDH*) of two independent experiments are shown. The expression level of *NANOG* in the control sample (DMSO) was set to 1 in each experiment.

B. Scatter plots showing the correlation of *NANOG* expression levels in *OSKM*-HCC827 cells and the effect on cell numbers in HCC827 cells, following treatment with test compounds. The expression level of *NANOG* (normalized to *GAPDH* or *mOct4*) in the control sample (DMSO) was set to 1.

C. Scatter plots showing the correlation of *NANOG* expression levels in *OSKM*-A549 cells and the effect on cell numbers in A549 cells, following treatment with test compounds. The expression level of *NANOG* (normalized to *GAPDH* or *mOct4*) in the control sample (DMSO) was set to 1.

Fig. S7. Uncovering a driver oncogenic pathway in clear cell sarcomas.

A. Derivation of *OSKM*-inducible human CCS cell lines. mCherry fluorescence reveals the expression of transgenes after Dox treatment in *OSKM*-induced MP-CCS-SY and KAS cells. Scale bars: 300 μm.

B. Knockdown efficiency for *EWS/ATF1* fusion gene in *OSKM*-MP-CCS-SY and *OSKM*-KAS cells. Data are presented as the mean ± SD of biological triplicates in RT-qPCR analysis. The expression level of *siControl*-treated cells in each experiment was set to 1.

C. A screen of MP-CCS-SY (left) and KAS (right) with 361 compounds in the SCADS inhibitor kit. Average values of *NANOG* expression (normalized to *mOct4*) of two independent experiments are shown. The expression level of *NANOG* in the control sample (DMSO) was set to 1 in each experiment.

D. Venn diagrams for the number of compounds that augmented *NANOG* expression in *OSKM*-MP-CCS-SY and *OSKM*-KAS cells.

Fig. S8. Uncovering a mTOR signaling as a driver oncogenic pathway in clear cell sarcomas.

A. Knockdown efficiency for the *MTOR*, *RPTOR*, and *RICTOR* gene in MP-CCS-SY and KAS cells. RT-qPCR data are presented as the mean ± SD of biological triplicates in RT-qPCR analysis. The expression level of *siControl*-treated cells in each experiment was set to 1.

B. Western blot analysis revealed that the downstream effector of mTORC1 and mTORC2 were downregulated by knockdown of *EWS/ATF1* in CCS cells.

C. GSEA of upregulated genes through activation of mTORC1 complex. *siEWS/ATF1* treatment inhibited target genes of mTORC1 activation in MP-CCS-SY cells.

D. Left: RNA-seq analysis of *MTOR* expression following *siEWS/ATF1* treatment in MP-CCS-SY cells. Right: GSEA of upregulated genes through activation of mTORC1 complex. *MTOR* knockdown inhibited target genes of mTORC1 activation in MP-CCS-SY cells.

E. Heat map of z-scores of affected genes (fold change >2) following *siEWS/ATF1* treatment in MP-CCS-SY cells. Z-scores in *siMTOR*-treated MP-CCS-SY cells are shown in the middle.

F. A Dox-controllable *piggyBac* vector for induction of an active mutant of mTOR (*PB-active MTOR* all-in-one). Hyperactive mutant of rat mTOR kinase ($mTOR^{SL1+IT}$), which is tagged with the FLAG epitope at its N terminus (Ohne *et al.*, 2008) was cloned into a Dox-inducible *piggyBac* vector.

G. Western blot analysis for the phosphorylation of S6RP. The phosphorylation of S6RP is increased in *siEWS/ATF1*-treated CCS cells following induction of the *active MTOR* gene.

H. Cell proliferation/survival assay in *siEWS/ATF1*-treated CCS cells following induction of the *active MTOR* gene.

I. Representative images of phosphorylated S6RP immunostaining for subcutaneous tumors in MP-CCS-SY xenografts. Scale bars: 500 μm (left), 100 μm (right).

Fig. S9. Identification of an effective combinatorial treatment with mTOR inhibitors in clear cell sarcomas.

A. RT-qPCR analysis for *NANOG* expression in *OSKM*-MP-CCS-SY cells (left) and *OSKM*-KAS cells (right) after the combinatorial treatment with rapamycin (test compounds: lower concentration of either IC$_{50}$ or 1 μM; rapamycin: 5 nM; Dox: 0.5 μg/ml; 48 hour treatment; 12-well plate). Data are presented as means $\pm$ SD of three independent experiments. The expression levels of *NANOG* were normalized by *GAPDH* (left) and *mOct4* (right). The mean value of DMSO-treated cells was set to 1 in each experiment.

B. RT-qPCR analysis for *NANOG* expression in *OSKM*-KAS cells after the combinatorial treatment with rapamycin (test compounds: 10 μM; rapamycin: 5 nM; Dox: 0.5 μg/ml; 48 hour treatment; 12-well plate). Data are presented as means $\pm$ SD of three independent experiments. The expression level of *NANOG* was normalized by *GAPDH* (left) and *mOct4* (right). The value of DMSO-treated cells was set to 1 in each experiment. ***P<0.001, *P<0.05; one-way ANOVA, followed by the Dunnett's *post-hoc* test.

C. The combinatorial treatment with rapamycin and p38 inhibitors augmented *NANOG* expression in *OSKM*-KAS cells (Dox: 0.5 μg/ml; 48 hour treatment; 6-well plate). Data are presented as the mean of three independent experiments. The expression level of *NANOG* in DMSO-treated cells in each experiment was set to 1.

D. RT-qPCR analysis for *NANOG* expression in human CCS cell lines after treatment with various p38 inhibitors (10 μM; Dox: 0.5 μg/ml; 48 hour treatment; 6-well plate). Data are presented as means $\pm$ SD of three independent experiments. The value of DMSO-treated cells was set to 1 in each experiment.

E. Knockdown efficiency for the *MAPK14* (p38α) gene in MP-CCS-SY and KAS cells. RT-qPCR data are presented as the mean ± SD of biological triplicates in RT-qPCR analysis. The expression level of *siControl*-treated cells in each experiment was set to 1.

F. RT-qPCR analysis for *NANOG* expression in *OSKM*-KAS cells after treatment with siRNAs targeting *MAPK14* (p38α) (Dox: 0.5 μg/ml; 48 hour treatment; 6-well plate). Data are presented as the mean of three independent experiments. The expression level of *NANOG* in DMSO/*siControl*-treated cells in each experiment was set to 1.

G. Cell proliferation/survival assay of CCS cells treated with p38 inhibitors.

H. Cell proliferation/survival assay of CCS cells treated with *siMAPK14* in combination with rapamycin.

Fig. S10. Identification of an effective combinatorial treatment with mTOR inhibitors in clear cell sarcomas.

A. Relative tumor volumes of mice bearing KAS xenografts treated with DMSO, rapamycin (2.5 mg/kg body weight), BIRB796 (50 mg/kg body weight), or both drugs in combination for the indicated times. Data are presented as means ± SD. ***$P<0.001$, **$P<0.01$, *$P<0.05$; unpaired two-tailed t-test.

B. Body weight of mice bearing KAS xenografts. No significant difference is observed in the body weight.

C. Representative images of immunostaining for phosphorylated S6RP and phosphorylated p38 in subcutaneous tumors in MP-CCS-SY xenografts. Arrows indicate nuclear staining of phosphorylated p38. Scale bars: 100 μm.

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Methods

Transduction of *OSKM* or *MYOD1* in mouse CCS cells

G1297, a mouse CCS cell line, was established previously (Komura *et al.*, 2019; Yamada *et al.*, 2013). Retroviral vectors were used for transduction of reprogramming factors into G1297 cells. pMX-*OCT4*, pMX-*SOX2*, pMX-*KLF4*, and pMX-*cMYC* were obtained from Addgene (Takahashi *et al.*, 2007; Takahashi and Yamanaka, 2006). *MYOD1* was cloned into a pMX vector. After the transduction of the reprogramming factors, G1297 cells were cultured in ESC media supplemented with human recombinant LIF (Wako), 2-Mercaptoethanol (Invitrogen) and 50 µg/ml L-ascorbic acid (Sigma). *OSKM*-induced G1297 cells were seeded at $0.4 \times 10^6 - 0.9 \times 10^6$ cells on MEFs in a 6 cm dish. The number of iPSC-like colonies was determined by adjusting mean numbers in a randomly selected fixed area.

MEFs with inducible *EWS/ATF1* alleles

After blastocyst injection of ESCs with inducible *EWS/ATF1* alleles (Komura *et al.*, 2019), chimeric embryos were harvested at E13.5, and MEFs were derived from the eviscerated and decapitated embryos with 0.25% trypsin. Since the $Rosa26^{M2rtTA}$ knock-in allele contains the *PGK-Puro-pA* selection cassette (Beard et al., 2006), MEFs were treated with 1 µg/ml puromycin (Sigma) for 1 week. The puromycin-resistant MEFs were used for further experiments.

FACS analyses

Cells were trypsinized, incubated with labeled antibodies together with propidium iodide, and analyzed with LSR Fortessa (BD biosciences). The Alexa Flour 647 conjugated anti-SSEA1 antibody (Santa Cruz, AF647 clone MC480, sc-21702; or BD Pharmingen, Clone MC480, 562277) was used to detect early reprogrammed cells.

Cell culture and establishment of *OSKM*-inducible cell lines

HCC827 and SK-BR3 cell lines were purchased from ATCC. PANC1 and K562 cell lines were purchased from DS Pharma Biomedical Co., Ltd.. The A549 cell line was obtained from RIKEN

BRC. The MP-CCS-SY cell line was provided by H. Moritake (University of Miyazaki) (Moritake et al., 2002). The KAS cell line was provided by T. Nakamura (Cancer Institute, Japanese Foundation for Cancer Research). The cell lines were grown in DMEM (Nacalai Tesque) supplemented with 10% of FBS (Biosera) and penicillin-streptomycin (PS) (Nacalai Tesque) for HCC827, SK-BR3, A549, MP-CCS-SY, and KAS, or RPMI1640 (Nacalai Tesque) supplemented with 10% FBS and PS for K562, or RPMI / F12 (Nakarai Tesque) supplemented with 13% FBS and PS for LCC-028 at 37 °C under 5% CO_2. Collagen-coated plates were used for SK-BR3, MP-CCS-SY, KAS and LCC-028. For establishment of *OSKM*-inducible cancer cell lines, a Dox-inducible *OSKM*-expressing vector (murine *OSKM*, KW394) and a PB transposase expression vector (KW158) (Woltjen *et al.*, 2009; Yagi *et al.*, 2019) were co-transfected into cancer cell lines by Lipofectamine 2000, Lipofectamine 3000 or Lipofectamine LTX reagent (Life Technologies), and the neomycin-resistant populations were selected (1 mg/ml). After Dox treatment, the expression of mCherry in each clone was confirmed under the BZ-X700 microscope (Keyence).

Patient-derived cells (PDCs)

LCC-028-3 cells harboring an *EML4-ALK* fusion gene were derived from pleural effusion of a patient with non-small cell lung cancer who exhibited a marked clinical response to treatment with alectinib (Sakamoto *et al.*, 2011). LCC-028-4 and LCC-028-5 cells were derived from pleural effusion of the same patient after acquisition of clinical resistance to alectinib (Sakamoto *et al.*, 2011). The patient-derived samples were obtained by a protocol approved by the institutional review board of the Japanese Foundation for Cancer Research (JFCR), following written informed consents were obtained from the patients. The clinical information from the patient medical records was reviewed.

Reverse transcription and quantitative PCR

Dox-controllable *OSKM*-expressing cancer cells were seeded in culture medium as described above. After 2–3 days of incubation with Dox (2 µg/ml for HCC827, SK-BR3, and A549; 0.5 µg/ml for K562, MP-CCS-SY, KAS, and LCC-028) and each compound, RNA was extracted by using the RNeasy Plus Mini Kit (QIAGEN) or NucleoSpin RNA Plus (Takara Bio). Up to 100 ng

RNA was used for the reverse transcription (RT) reaction into cDNA. Quantitative PCR was performed using the Go-Taq qPCR Master Mix (Promega) and analyzed on a StepOnePlus Real-Time PCR system (Applied Biosystems). Transcript levels were normalized to *β-actin* (for mouse cells), *GAPDH* (for human cells) or transgenic m*Oct4* (for human cells). The sequences of the primers used for RT-qPCR analyses were as follows: m*Oct4* forward, GTGGAGGAAGCCGACAACA; m*Oct4* reverse, ACTCCACCTCACACGGTTCT; m*Sox2* forward, AACGCCTTCATGGTATGGTC; m*Sox2* reverse, CGGACAAAAGTTTCCACTCC; m*Klf4* forward CTGAACAGCAGGGACTGTCA; m*Klf4* reverse, GAGGGGACTTGTGACTGCAT; m*Myc* forward, ACACGGAGGAAAACGACAAG; m*Myc* reverse, AATTCAGGGATCTGGTCACG; *Nanog* forward, TGCTTACAAGGGTCTGCTACTG; *Nanog* reverse, TAGAAGAATCAGGGCTGCCTTG; *β-actin* forward, GCCAACCGTGAAAAGATGAC; *β-actin* reverse, TCCGGAGTCCATCACAATG; *Cdh1* forward, TCCTGCCAATCCTGATGAAA; *Cdh1* reverse, AACCACTGCCCTCGTAATCG; *Myog* forward, GAGACATCCCCCTATTTCTACCA; *Myog* reverse, GCTCAGTCCGCTCATAGCC; *NANOG* forward, TCCAACATCCTGAACCTCAGC; *NANOG* reverse, TTCGGCCAGTTGTTTTTCTGC; *GAPDH* forward, ATGGGGAAGGTGAAGGTCG; *GAPDH* reverse, GGGGTCATTGATGGCAACAATA; type1 *EWS/ATF1* forward, GAGGCATGAGCAGAGGTGG; type1 *EWS/ATF1* reverse, GAAGTCCCTGTACTCCATCTGTG; type2 *EWS/ATF1* forward, GACCCATGGATGAAGGACCA ; type2 *EWS/ATF1* reverse, ACCTTGCTGAGTACTGCCTG ; *EGFR* forward, GCCTTGACTGAGGACAGCAT; *EGFR* reverse, GGGCTGGACAGTGTTGAGAT; *FGFR* forward, ATGCAGTGCCCTCACAGAG; *FGFR* reverse, CAGCGGCTCATGAGAGAAG ; *MTOR* forward, GACGAGAGATCATCCGCCAG; *MTOR* reverse, ACAAGGGACCGCACCATAAG; *RPTOR* forward, CGGCTGACCTATTCACCTCC; *RPTOR* reverse, CAGGCACCAGACTGACACAT; *RICTOR* forward, GGGGTGTCTCAAGAAGGCTC; *RICTOR* reverse, GCGAAGGAGTATACGGCACA.

RT-qPCR for *NANOG* expression in 96-well cell culture plates

Dox-controllable *OSKM*-expressing cancer cells were seeded into 96-well cell culture plates

(5,000 cells/well/100 µl, Thermo Fisher Scientific) in culture medium as described above. Twenty-four (HCC827, A549, and LCC-028) or 72 hours (MP-CCS-SY and KAS) after incubation, the media was changed by to the media containing Dox (2 µg/ml for HCC827 and A549; 0.5 µg/ml for MP-CCS-SY, KAS, and LCC-028) and each compound, and the assay plates were further incubated. After 2–3 days of incubation, RNA extraction and RT-qPCR analysis were performed using TaqMan Gene Expression Cells-to-CT Kit or Fast Advanced Cells-to-CT kits (Thermo Fisher Scientific) and QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems) or QuantStudio 5 Real-Time PCR System (Applied Biosystems).

The sequences of the primers and TaqMan probes used for RT-qPCR analyses in 96-well plates were as follows: *NANOG* forward, TCCAACATCCTGAACCTCAGC; *NANOG* reverse, TTCGGCCAGTTGTTTTTCTGC; *NANOG* probe, FAM-CCAGAGAATGAAATCTA-MGB; mouse-*Oct4* forward, GTGGAGGAAGCCGACAACA; mouse-*Oct4* reverse, ACTCCACCTCACACGGTTCT; mouse-*Oct4* probe, VIC-AACCTTCAGGAGATATGCAAA-MGB; *GAPDH* forward, ATGGGGAAGGTGAAGGTCG; *GAPDH* reverse, GGGGTCATTGATGGCAACAATA; *GAPDH* probe, ABY-CGCCTGGTCACCAGGGCTGCT-QSY.

Western blot analysis

Cultured cells were harvested in 500 µl of RIPA lysis buffer, and protein concentration was measured. Proteins were denatured with 6×SDS in 95 ℃ for 5minutes. A total of 20 µg denatured protein was applied to a 10% SDS/PAGE gel and transferred to a PVDF membrane (Amersham Hybond-P PVDF Membrane, GE Healthcare). Proteins were detected by immunoblotting with the following antibodies: anti-EGFR (abcam#ab52894; dilution 1:1200), anti-phosho-S6 Ribosomal Protein (Ser235/236) (Cell Signaling#2221; dilution 1:1000), anti-S6 Ribosomal Protein (Cell Signaling#2217; dilution 1:1000), anti-phosho-4EBP1 (Thr37/46) (Cell Signaling#2855; dilution 1:1000), anti-4EBP1 (Cell Signaling#9644; dilution 1:1000), anti-phosho-Akt (Ser473) (Cell Signaling#4060; dilution 1:1000), anti-Akt (Cell Signaling#4691; dilution 1:1000), anti-β actin (Santa Cruz#sc-47778; dilution 1:1000). Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific) was used for visualization, and LAS4000 and

Amersham Imager 680 (GE Healthcare) were used for detection.

Chemical libraries

The SCADS inhibitor kit was provided by the Molecular Profiling Committee in Advanced Animal Model Support (AdAMS). The 619 kinase inhibitors were provided by the drug discovery technology development office at CiRA. All chemical compounds were dissolved in DMSO at a final concentration of 100 µM and stored at −80°C when not in use. Other reagents were purchased from Selleckchem or Cayman Chemical and dissolved in DMSO for cell culture experiments.

siRNA transfection

Gene-specific scrambled siRNA pools were purchased from Dharmacon. For *EWS*/*ATF1* fusion gene knockdown, siRNAs targeting the breakpoint of *EWS*/*ATF1* type1 (sense, GCGGUGGAAUGGGAAAAAUTT; antisense, AUUUUUCCCAUUCCACCGCTT) (AteloSiLence) were used for MP-CCS-SY. siRNAs targeting the breakpoint of *EWS*/*ATF1* type2 (sense, CUUGAUCUAGUUGCCAUUGCTT; antisense, GCAAUGGCAACUAGAUCAAGTT) (AteloSiLence) were used for KAS. Lipofectamine RNAi Max reagent (Thermo Fisher Scientific) was used for the siRNA transfection.

Cell proliferation/survival assays

For cell proliferation/survival assays upon drug treatments, cells were incubated with the WST-8 assay using a Cell Counting Kit-8 (Dojindo Laboratories) for 45 minutes, and the absorbance was measured at 450 nm using a microplate reader following drug treatments for 48–96 hours. For survival assay in Fig. 5D, the data were graphically displayed using GraphPad Prism version 7.04 (GraphPad Software). The IC_{50} value was determined by a nonlinear regression model with a sigmoidal dose-response in GraphPad.

Clonogenic assay

For clonogenic assays, cells were seeded into 6-well plates (5×10^4 to 15×10^4 cells/well) and

allowed to adhere overnight. Cells were then cultured in the absence or presence of drugs as indicated in complete media for 6–8 days. Growth media with or without drug was replaced every 2 or 3 days. The remaining cells were fixed with 4% paraformaldehyde and then stained with 0.5% crystal violet. All experiments were performed at least three times. Representative experiments are shown.

Library preparation for RNA sequencing (RNA-seq)

Total RNA was isolated using the NucleoSpin RNA Plus (Takara Bio). RNA was quantified on a NanoDrop 2000c and Qubit. RNA (200 ng) was prepared for library construction. High-quality RNA (RNA Integrity Number value ≥8, as determined by Bioanalyzer) was used for library preparation. RNA-seq libraries were generated using the NEBNext Ultra II Directional RNA Library Prep kit for Illumina (NEB). The number of PCR cycles was minimized to avoid skewing the representation of the libraries. RNA-seq libraries were sequenced on a NextSeq500 (Illumina) as 86 bp single reads.

RNA-seq data analyses

The sequenced reads were trimmed to remove low-quality bases and adaptor sequences using cutadapt-1.18 (Martin, 2011). The trimmed reads were mapped to the mouse reference genome (mm10) or the human reference genome (hg38) using STAR v2.6.0c with GENCODE vM25 (mouse) or v36 (human) (Frankish et al., 2019). The uniquely mapped reads were summarized at the gene level using HTSeq-count v0.11.2 (Anders et al., 2015), and the expression level of each gene was calculated as TPM (transcripts per million) using DESeq2 v1.30.0 (Love et al., 2014).

ChIP-sequencing

Anti-HA antibody (Nacalai Tesque, mouse monoclonal, HA124), anti-V5 antibody (Abcam, rabbit polyclonal, ab15828), and anti-OCT4 antibody (CST, rabbit polyclonal, #2750) were used for the ChIP-seq analysis. ChIP experiments were performed as described previously (Hinohara et al., 2018), with the minor modification that ChIP-ed DNA was purified with AMPureXP (Agencourt) at the last step. Sequencing libraries were prepared using ThruPLEX DNA-Seq Kit

(TaKaRa), quantified with TapeStation (Agilent), and subjected to paired-end sequencing (75bp x2) with NextSeq 550 (Illumina). Adaptor sequences in reads were trimmed using Trim Galore version 0.5.0 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). The trimmed reads were aligned to the mm9 genome build using bowtie2 version 2.3.4.3 with default parameters except the --very-sensitive option, and filtered based on mapping scores (MAPQ ≥30) by samtools version 1.9 (Langmead and Salzberg, 2012; Li et al., 2009). Duplicated reads were removed using picard MarkDuplicates version 2.18.7. Reads mapped to regions blacklisted by ENCODE were removed with bedtools (Quinlan and Hall, 2010). MACS2 version 2.1.2 was used to identify peaks in each sample with default settings (Zhang et al., 2008). All the peaks were then merged into a single reference peak list using bedtools merge, and the number of reads that fell into each peak was counted using bedtools multicov. Normalization of the count data was performed by using DESeq2. Motif analyses were performed using HOMER with default settings (Heinz et al., 2010).

Xenografts

All animal studies were approved by the IMSUT Animal Experiment Committee, and the care of the animals was in accordance with institutional guidelines. 5- to 7-week-old female BALB/cSLC-nu/nu nude mice (Japan SLC) were used for animal experiments with human cell lines. For MP-CCS-SY and KAS xenografts, 5×10^6 cells were harvested on the day of use and injected with a growth-factor-reduced Matrigel/PBS solution (30% final concentration). After inoculation, mice were monitored daily, and caliper measurements began when tumors became visible. Tumor volume was calculated using the following formula: tumor volume = $(D \times d^2)/2$, in which D and d refer to the longest and shortest tumor diameter, respectively. Mice were randomized into cohorts and treated with vehicle, Rapamycin (2.5 or 5.0 mg/kg, i.p., every day), BIRB796 (50 mg/kg, oral gavage, every 2 days), or a combination. Rapamycin was dissolved in the solution (16% DMSO, 30% Polyethylene glycol 400, 5.0% Tween80), and BIRB796 was dissolved in a ethanol/water solution (60:40).

Statistics

The statistical significance of all experiments was calculated using 2-tailed Student's t-test or one-way ANOVA for Dunnett's multiple comparisons t-test. All statistical test was performed using GraphPad Prism 7.04 (GraphPad Software). P-values of<0.05 were considered as statistically significant.