

De novo mutations in *MSL3* cause an X-linked syndrome marked by impaired histone H4 lysine 16 acetylation

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The etiological spectrum of ultra-rare developmental disorders remains to be fully defined. Chromatin regulatory mechanisms maintain cellular identity and function, where misregulation may lead to developmental defects. Here, we report pathogenic variations in *MSL3*, which encodes a member of the chromatin-associated male-specific lethal (MSL) complex responsible for bulk histone H4 lysine 16 acetylation (H4K16ac) in flies and mammals. These variants cause an X-linked syndrome affecting both sexes. Clinical features of the syndrome include global developmental delay, progressive gait disturbance, and recognizable facial dysmorphism. *MSL3* mutations affect MSL complex assembly and activity, accompanied by a pronounced loss of H4K16ac levels in vivo. Patient-derived cells display global transcriptome alterations of pathways involved in morphogenesis and cell migration. Finally, we use histone deacetylase inhibitors to rebalance acetylation levels, alleviating some of the molecular and cellular phenotypes of patient cells. Taken together, we characterize a syndrome that allowed us to decipher the developmental importance of *MSL3* in humans.

Recent advances in whole-exome sequencing have led to the discovery of numerous causative genes for human diseases. However, gathering a large number of patients represents a sig-

nificant bottleneck for gene identification and appropriate molecular analysis of rare developmental diseases. Mutations of epigenetic regulators were found to cause neurodevelopmental disorders through

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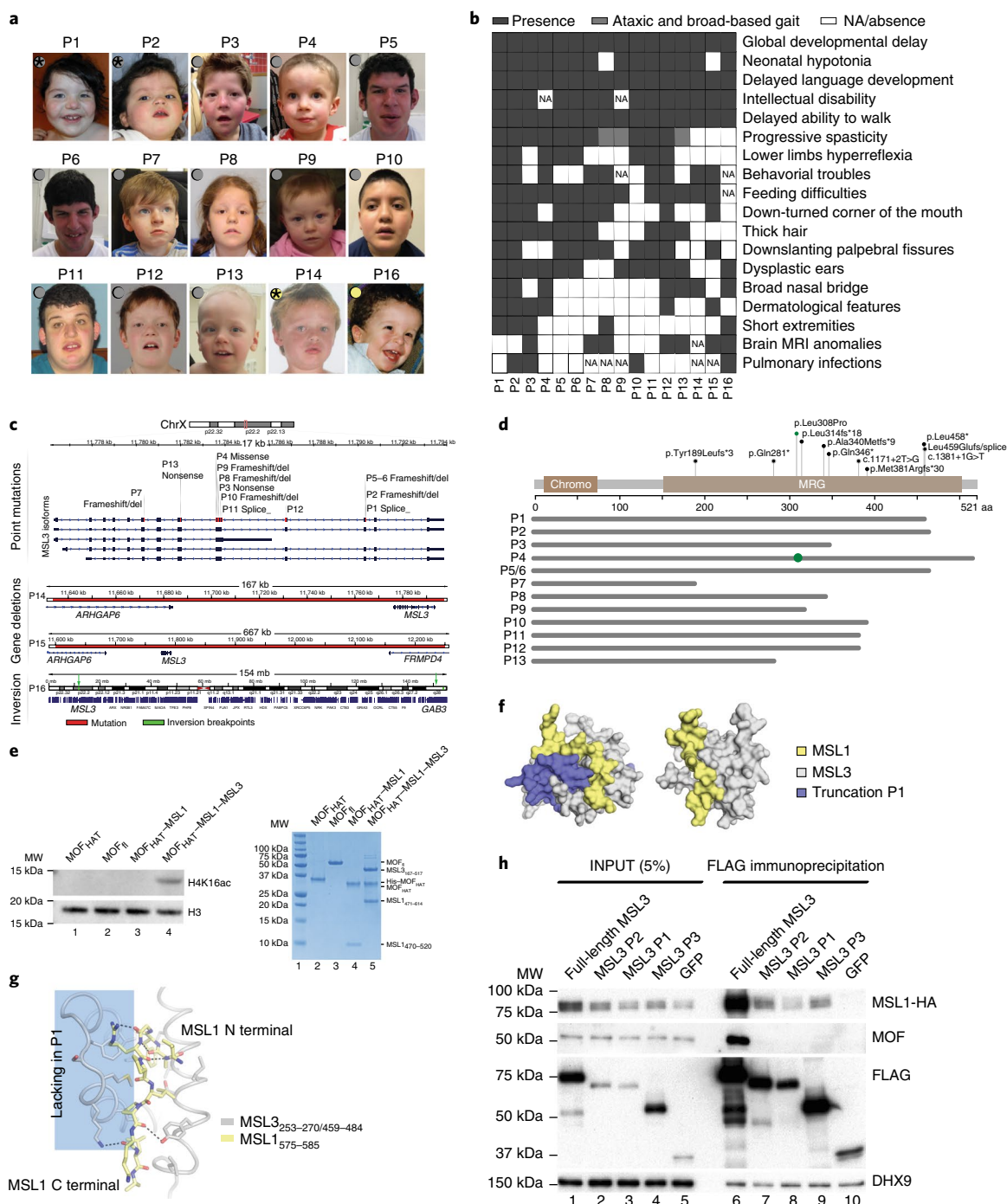


Fig. 1 | Mutations in *MSL3* cause a novel neurodevelopmental disorder. a, Photographs of patients with *MSL3* mutations. Gray circles indicate intragenic variants. Yellow circles indicate whole-gene deletions. Stars show the patients who donated skin biopsies. Written consent for the use of photographs was obtained from the parents of affected individuals. **b**, Human Phenotype Ontology heat map of patients' common clinical features. Blank boxes represent either absent or unreported symptoms. MRI, magnetic resonance imaging; NA, not analyzed. **c**, The *MSL3* locus and identified variants are highlighted in red. P1–P13 variants occur within *MSL3* (top). P13/P14 deletions span *ARHGAP6*, *MSL3*, and *FRMD4* (middle). P16 chromosomal inversion and breakpoints (green) are between Xp22.2 and Xq28 (bottom). **d**, Annotation of the pathogenic variants on the primary protein structure. Gray lines represent predicted proteins, where most SNVs (except for p.Leu308Pro; shown as green circle) result in MRG domain truncation. aa, amino acid. fs, frameshift. **e**, Left, cropped H4K16ac and H3 immunoblot of a HAT assay performed on nucleosomal substrate with the indicated proteins (full-length (fl; 2–458) and HAT domain (174–458)). Right (lanes 2–5), Coomassie-stained gel of the assayed proteins. The experiment was repeated twice with similar results. MW, molecular weight (lane 1). **f**, Modeled surface representation of the MSL1–MSL3 complex (Protein Data Bank: 2YON; MSL1, yellow; MSL3, gray). Missing parts in P1 are highlighted in purple (left) or removed (right). **g**, Schematic representing the MSL1 (575–585, yellow) and MSL3 (253–270/459–484, gray) interaction interface. Hydrogen bonds are shown in black. α -helices 5/6, which are missing in P1, are shaded blue. **h**, FLAG co-immunoprecipitations of FLAG-tagged full-length MSL3, MSL3 variants (P2, P1, and P3) and a GFP control with cropped immunoblots. MSL3-FLAG-tag, MSL3(P2,P1,P3)-FLAG-tag, GFP-FLAG-tag expression vectors were transiently co-transfected with MSL1-HA-tag expression vector into Flp-In T-Rex 293 cells. DHX9 serves as input loading control. The experiment was repeated four times with similar results.

Table 1 | *MSL3* variants identified in the 16 individuals from this study

Patient identification	Genomic position hg19	cDNA NM_078629.3	Protein Q8N5Y2-1	Inheritance
P1	chrX:g.11790375G>T	c.1381+1G>T	NA	De novo
P2	chrX:g.11790367_11790374del	c.1374_1381del	p.(Leu459Glufs*13)	De novo
P3	chrX:g.11783713C>T	c.1036C>T	p.(Gln346*)	De novo
P4	chrX:g.11783600T>C	c.923T>C	p.(Leu308Pro)	De novo
P5	chrX:g.11790365C>T	c.1372C>T	p.(Arg458*)	De novo (parental mosaicism)
P6	chrX:g.11790365C>T	c.1372C>T	p.(Arg458*)	De novo (parental mosaicism)
P7	chrX:g.11780349_11780350delAC	c.566_567delAC	p.(Tyr189Leufs*3)	De novo
P8	chrX:g.11783695delG	c.1018delG	p.(Ala340Leufs*9)	De novo
P9	chrX:g.11783615dupC	c.938dupC	p.(Leu314Phefs*18)	De novo
P10	chrX:g.11783802_11783818dup17	c.1125_1141dup17	p.(Met381Argfs*30)	De novo
P11	chrX:g.11783850T>G	c.1171+2T>G	NA	De novo
P12	Del exon 10	.	.	De novo
P13	chrX:g.11781990C>T	c.841C>T	p.(Gln281*)	De novo
P14	chrX:g.11633731_11797224del	.	.	De novo
P15	chrX:g.11600773_12249902del	.	.	NA (father not tested; absent in the mother)
P16	Inv(X)(p22.2q28)	.	.	Inherited from the mother (de novo in the mother)

Del, deletion; dup, duplication; NA, not available; fs, frameshift; Inv(X), inversion (X chromosome)

mechanisms potentially altering the chromatin landscape¹. To date, over 40 Mendelian disorders have been identified that are caused by mutations of the epigenetic machinery and associated with neurological dysfunction, 80% of which are autosomally encoded^{2–4}. Fluctuations in the histone acetylation balance, due to the interplay between acetylase and deacetylase functions during development, contributes to the final shape and performance of the adult brain and the whole organism^{5–8}. While several modifications within the histone core were found to alter the chromatin architecture⁹, histone H4 lysine 16 acetylation (H4K16ac) appears uncommon among the tail modifications as it directly affects higher-order chromatin structure¹⁰. The male-specific lethal (MSL) complex-associated histone acetyltransferase KAT8, also known as MOF (males-absent on the first protein), is responsible for bulk H4K16ac in flies and mammals¹¹. Given its crucial importance, we know surprisingly little about the role of MSL-mediated H4K16ac during development and whether its misregulation is associated with any disease in humans.

Results

Mutations in *MSL3* cause a neurodevelopmental disorder. Through the international Matchmaker Exchange initiative¹², we have identified de novo variants in MSL homolog 3 (*MSL3*; previously known as *MSL3L1*) RefSeq gene NG_012564.1) in 15 unrelated families from Europe, the USA, and Australia (Fig. 1a,b and Supplementary Fig. 1a). In the matchmaking cohort, we identified 11 truncating variants (P1–3, P5–11, and P13), one exon deletion (P12), one missense variant (P4), two gene deletions (P14–15) and one chromosomal inversion (P16) (Fig. 1c and Table 1). Patients 1–9 were gathered from a cohort of approximately 10,000 individuals with intellectual disability, demonstrating a significant enrichment for *MSL3* de novo truncating variants in this cohort compared with random occurrence (uncorrected $P=8.98 \times 10^{-16}$; false discovery rate (FDR) $P=1.64 \times 10^{-11}$) (Supplementary Fig. 1b,c and Table 1). The enrichment for truncating variants in this cohort of patients is consistent with the data from the Exome Aggregation Consortium (ExAC) database, suggesting that *MSL3* is extremely intolerant to loss-of-function variations (probability of being loss-of-function intolerant (pLI) = 0.95)¹³. For patients 5 and 6

(monozygotic twins), deep sequencing of leucocyte parental DNA did not detect an alternative allele. Patient 16 inherited the X-inversion, causing a putative in-frame transcript between *MSL3* and *GAB3* from his mildly affected mother (Table 1 and Supplementary Fig. 1d).

MSL3 is known for its role during X chromosome dosage compensation via H4K16ac in *Drosophila*. Its absence affects male flies and manifests in lethality in the last larval stage of development^{11,14,15}. The human *MSL3* gene resides on the X chromosome and its mutations presented here affect both hemizygous male and heterozygous female individuals (Fig. 1a and Supplementary Table 1). Common features across the 16 patients include hypotonia, feeding difficulties in early infancy, and global delay in the acquisition of major milestones including walking and speech. Intellectual disability was also scored. The phenotypic spectrum is more homogeneous across patients of both sexes with single nucleotide variants (SNVs) compared with patients with chromosomal rearrangements. The patients with SNVs display progressive spasticity or an ataxic gait (9/13), resulting in the inability to walk (5/13). The facial morphology strongly overlaps across patients with telecanthus (8/13), epicanthal folds (9/13), down-slanting palpebral fissures (9/13), downturned corners of the mouth (8/13), and dysplastic ears (11/13) (Fig. 1b and Supplementary Table 1; see also the detailed clinical descriptions in Supplementary Note 1). Together, these clinical observations delineate a recognizable *MSL3* syndrome. In line with these phenotypes, *MSL3* is ubiquitously expressed in the developing human brain until 17–20 weeks post-conception. Other MSL complex members display a similar expression pattern, although their RNA levels seem slightly higher (Supplementary Fig. 1e)¹⁶.

Most of the pathogenic *MSL3* variants affect the carboxy (C)-terminal MRG domain (Fig. 1d and Supplementary Fig. 1f), which is responsible for MSL complex formation while enhancing the activity of MOF towards H4K16ac (Fig. 1e)^{17,18}.

Using in silico modeling of the crystal structure of the human *MSL3*–*MSL1* complex¹⁷, we predicted that the truncations present in the patients impair the integration of mutant *MSL3* into the MSL complex (Fig. 1f,g). To test this, we transiently transfected expression vectors encoding wild-type (full-length) *MSL3* and three different *MSL3* mutants, representing patients 1, 2, and 3 (P1, P2, and P3),

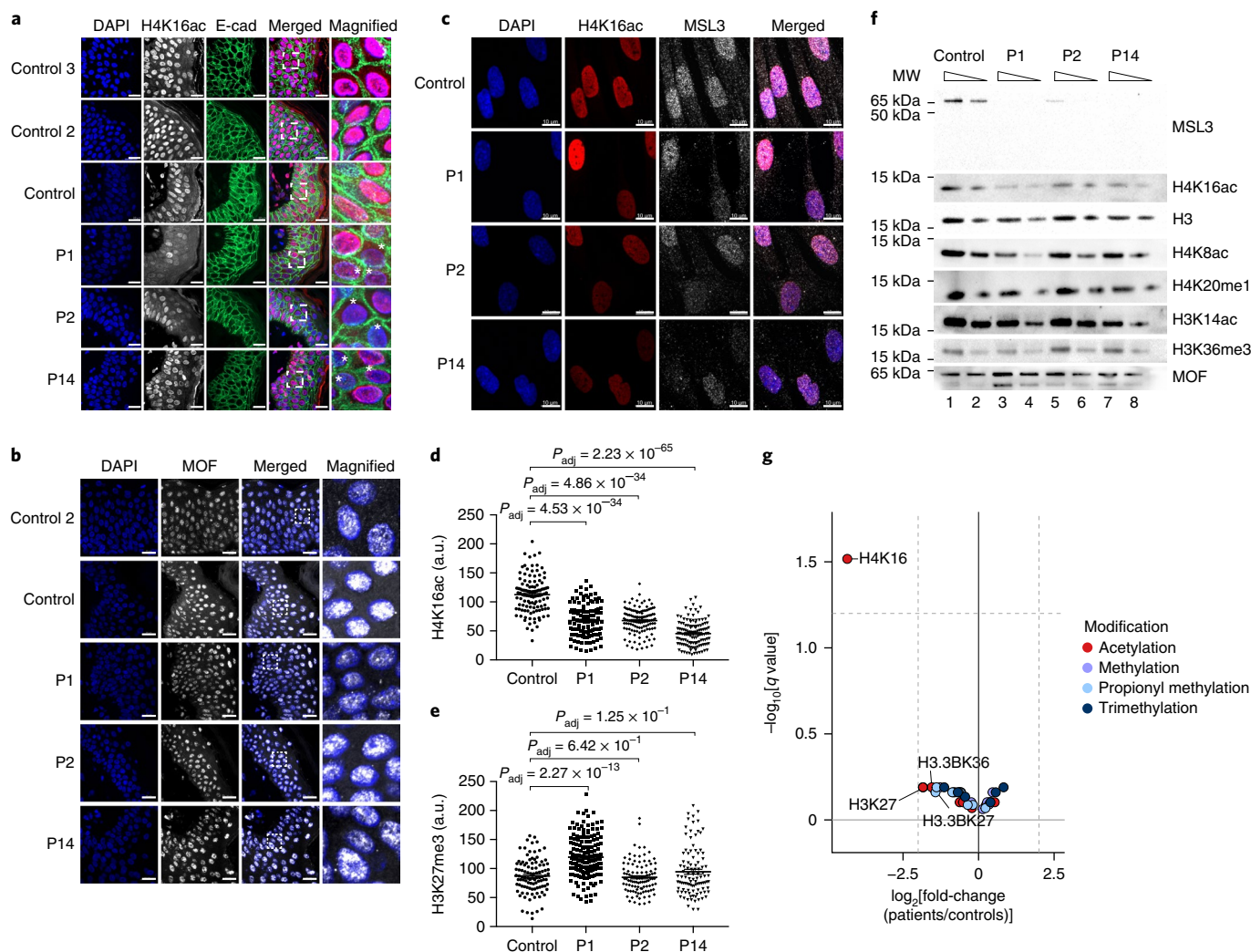


Fig. 2 | Bulk reduction of H4K16 acetylation in patient cells. a, Immunohistochemistry of FFPE skin sections for H4K16ac (white, but pink in the merged images) and E-cadherin (E-cad, green). 4',6-diamidino-2-phenylindole (DAPI) staining is shown in blue. Asterisks highlight cells with reduced H4K16ac staining. Scale bars = 20 μm , the pictures in the final column (Magnified) represent a 4.4x magnification of the merged column pictures. Staining was repeated twice with similar results. **b**, As in **a**, but for MOF (white). The pictures in the final column (Magnified) represent a 5.1x magnification of the merged column pictures. **c**, Immunostaining of the Control and P1-, P2-, and P14-derived primary HDFs for H4K16ac (red) and MSL3 (white). The staining was repeated twice ($n = 2$ sections per slide). **d, e**, Quantification of staining intensities for H4K16ac (**d**) and histone H3 lysine 27 trimethyl (H3K27me3) (**e**) from one representative experiment for the Control and P1, P2, and P14 HDFs. Each data point corresponds to the intensity of a single cell with the center line representing the mean \pm s.e.m. P values were determined by ordinary one-way analysis of variance followed by Bonferroni correction. **f**, Cropped immunoblot of HDF nuclear extracts. 100 and 50% dilutions were loaded. The experiment was reproduced three times. MW, molecular weight. **g**, Volcano plot of the indicated histone modification abundance displayed as $\log_2[\text{fold-change}]$ (x axis, P1, P2, and P14 HDFs ($n = 6$; 2 for each patient) compared with the Control HDFs ($n = 3$)) versus the statistical significance (y axis, two-sided, moderated t -test) as determined by LC-MS. Vertical dashed lines represent fourfold changes, and the horizontal dashed line shows a $-\log_{10}[q \text{ value}]$ of 1.2, Benjamini-Hochberg-corrected FDR of 0.05. Red circles show acetylated sites, violet circles show monomethylated sites, light blue circles show propionyl methylated sites, and dark blue circles show trimethylated sites. Detailed results of the statistical analyses related to **d** and **e** are provided in Supplementary Table 5, and those for **g** are presented in Supplementary Table 2.

into Flp-In T-REx 293 cells. We observed that in contrast with wild-type MSL3, which showed robust interaction with MSL1 and MOF, all three mutants lost the interaction with MOF and, to a lesser extent, with MSL1 (Fig. 1h, lanes 6–9). Two of the mutations also decreased MSL3 protein stability (Fig. 1h, lanes 2 and 3). Taken together, we find that the mutations present in the patients with MSL3 syndrome lead to severely compromised MSL complex integrity.

Bulk reduction of H4K16ac in cells from patients with MSL3 mutations. To characterize the molecular consequences of MSL3

mutations in patients, we obtained skin biopsies from two female patients harboring frameshift mutations (P1 and P2), one female patient with a locus deletion (P14), and one control tissue from P1's healthy mother (referred to as Control). In addition, we analyzed two more skin biopsies from healthy donors as controls (referred to as Control 2 and Control 3, see Methods). These samples were used to perform histological analyses and establish primary human dermal fibroblast (HDF) cell culture models for all molecular analyses presented below.

Hematoxylin and eosin staining confirmed that the overall tissue architecture was preserved in both Control and patient specimens

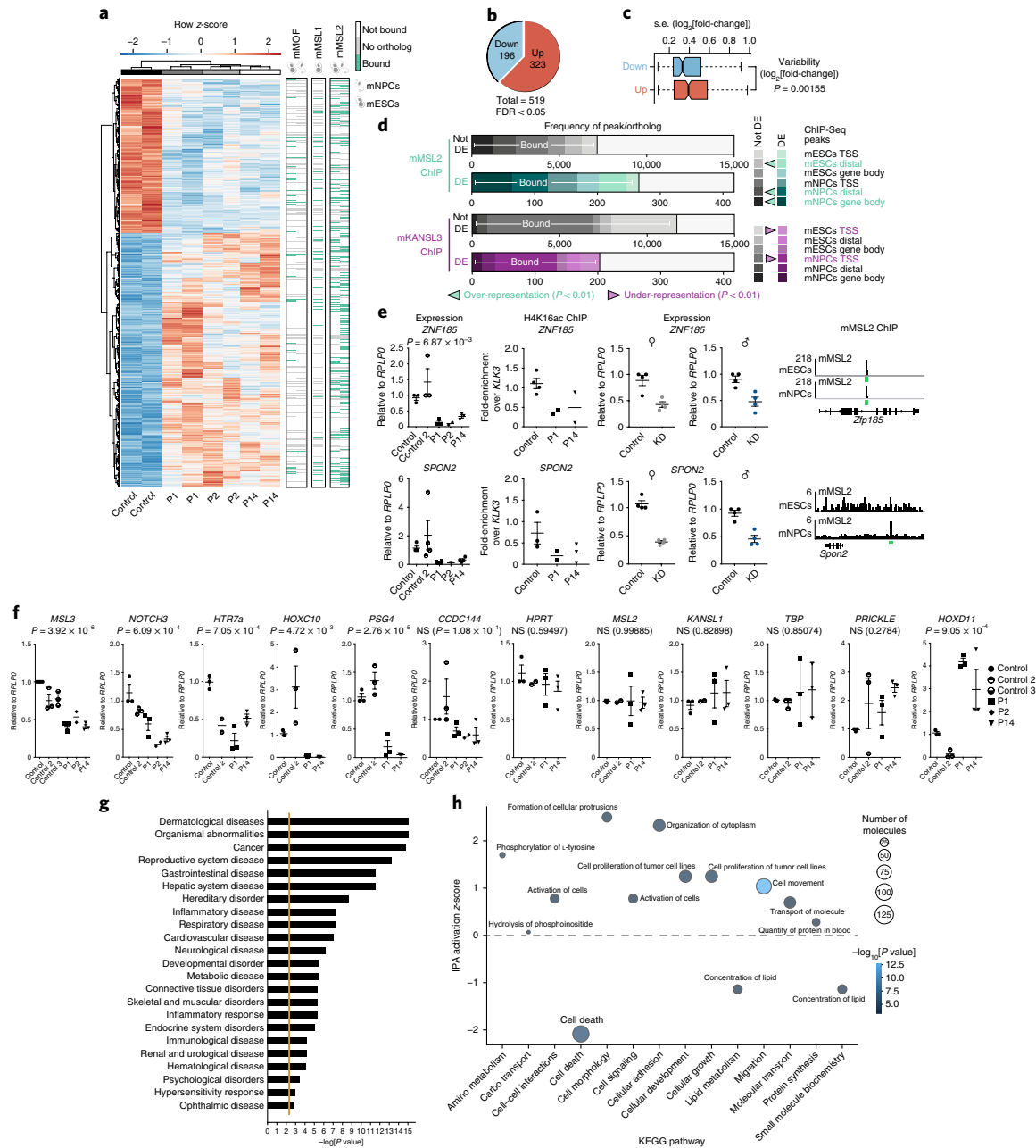


Fig. 3 | Loss of *MSL3* leads to transcriptome misregulation of developmental pathways. **a**, Left, Heat map showing the z-scores of differentially expressed (DE) genes (P1, P2, and P14 versus Control) obtained by RNA-Seq (FDR = 0.05). Right, MACS2 ChIP-Seq²¹ peaks for mouse *MSL1* (mMSL1), mouse *MSL2* (mMSL2) and mouse *MOF* (mMOF) at mouse orthologs of *MSL3* patient DE genes (green, bound; white no peak; gray, no ortholog) in mouse embryonic stem cells (mESCs) and mouse neural progenitor cells (mNPCs). **b**, Pie chart of DE genes. **c**, Box plot displaying DESeq2 log₂[fold-change] s.e. for the DE downregulated ($n = 196$) versus DE upregulated genes ($n = 323$). The P value was calculated using a two-sided Welch two-sample t -test. **d**, ChIP-Seq peak frequency plot for mouse mMSL2 or mouse KANSL3 (mKANSL3) at unaffected/not DE versus misregulated/*MSL3* DE genes in patients. P values for ChIP peak over-representation or under-representation within the groups were calculated using a one-sided Fisher's test. TSS, transcription start site. **e**, Dot plots (center line representing the mean \pm s.e.m.) of expression and H4K16ac ChIP-qPCR analyses. Expression levels were determined in controls and patients and on *MSL3* knockdown (KD) in male (δ) and female (ϕ) control HDFs. H4K16ac ChIP-qPCR enrichment values were calculated relative to input and expressed as the fold-change over *KLK3*. Right, genome browser snapshots of mMSL2 ChIP-Seq enrichment at corresponding mouse orthologs. The y-axis represents 1x sequencing depth normalized read densities (number of reads per bin/(total number of mapped reads * fragment length / effective genome size)). **f**, RT-qPCR expression analysis displayed as dot plots with the center line representing the mean \pm s.e.m. Expression levels were normalized to *RPLP0* and expressed relative to the Control. NS, not significant. P values were determined by ordinary one-way analysis of variance followed by Bonferroni correction. **g**, IPA z-score plot for the disease and function signature of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways ($P_{adj} < 0.005$ cut-off). Benjamini-Hochberg multiple testing correction was applied ($n = 519$ differentially expressed genes). The vertical gold line shows the threshold. **h**, Bubble plot representing perturbation pathway analysis for molecular and cellular functions. The bubble size shows the molecule abundance, while the color represents the IPA z-score (see scale bar). The most representative sub-pathway by number of molecules was selected to represent the full signature ($n = 477$ transcripts). For the dot plots in (e,f), each dot represents an independent experiment in HDFs. Further details and statistical test values for each plot are provided in Supplementary Table 5.

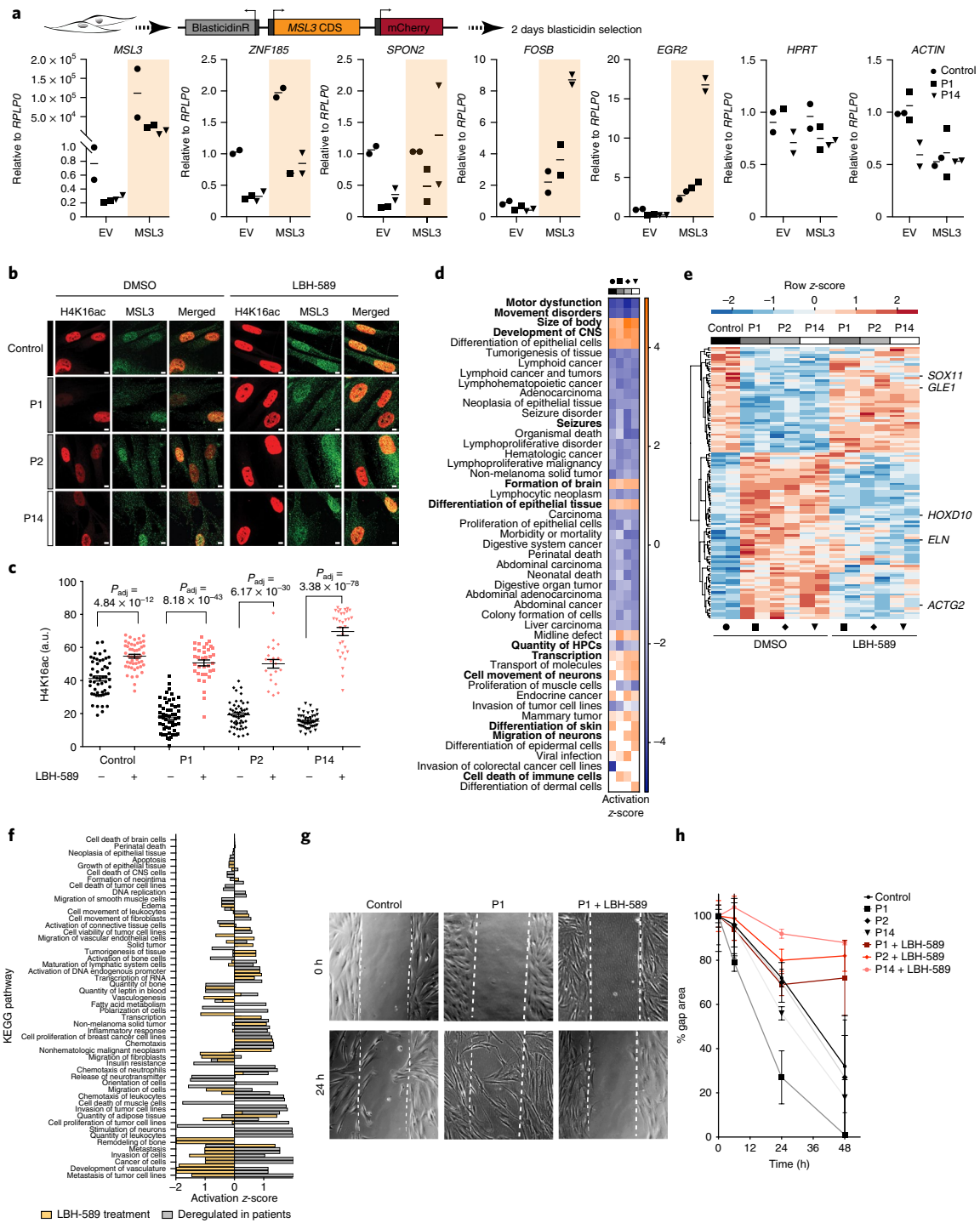


Fig. 4 | Histone deacetylase inhibition alleviates some cellular and molecular phenotypes in patient cells. a, *MSL3* and empty expression vector (EV) were transfected in Control and P1/P14 HDFs. Dot plots depict RNA levels from each independent experiment normalized to *RPLP0* relative to the EV. Center lines represent the mean ($n = 2$). CDS, protein coding sequence; BlasticidinR, blasticidin S resistance gene. **b**, Immunofluorescence for H4K16ac (red) and *MSL3* (green) on treatment with $4 \mu\text{M}$ LBH-589. Scale bar = $5 \mu\text{m}$. The staining was reproduced twice. **c**, Quantification of **b**. Data points represent H4K16ac intensities of individual cells from one representative experiment. Center lines represent the mean \pm s.e.m. *P* values were determined by ordinary one-way analysis of variance followed by Bonferroni correction. Detailed results of the statistical analyses are shown in Supplementary Table 5. **d**, Heat map of activation z-scores obtained from IPA (comparison analysis; disease and function signature ($n = 3,391$ for the Control; $n = 2,740$ for P1; $n = 2,487$ for P2; $n = 2,737$ for P14; $P_{adj} < 1 \times 10^{-4}$ for differentially expressed genes on LBH-589 treatment ($2 \mu\text{M}$)). Activated pathways were plotted for $|\pm z| > 2.5$ and a $\log_{10}[P \text{ value}]$ cut-off of 1.5. Bold type shows the pathways impacting on patient cell physiology. CNS, central nervous system. Black circle, Control; square, P1; rhombus, P2 and triangle, P14. **e**, Heat map representing z-scores of differentially expressed genes reverting to not differentially expressed on LBH-589 treatment ($n = 109$). Five disease-relevant transcripts are highlighted. Black circle, Control; square, P1; rhombus, P2 and triangle, P14. **f**, IPA comparison analysis of KEGG pathway z-scores in genes rescued on LBH-589 ($n = 109$ with an expression cut-off of $\log[\text{ratio}] > |\pm 0.2|$). **g**, Representative differential interference contrast images of HDFs at 0 and 24 h after creating a ‘scratch’ (gap area). **h**, Quantification of the percentage gap area at 0, 6, 24, and 48 h for Control and patient HDFs grown with or without LBH-589 ($2 \mu\text{M}$). The center line represents the mean \pm s.e.m. ($n = 3$).

(Supplementary Fig. 2a). The MSL complex is responsible for bulk H4K16ac in flies^{19,20} and mammalian cell culture models^{14,15,21}. Based on this, we performed immunostaining of H4K16ac in patient samples compared with skin punches of Controls. While the H4K16ac staining had homogeneous intensity in dermal and epidermal layers of all three control samples, *MSL3* patient cells showed a heterogeneous pattern with a marked and variable reduction in H4K16ac (Fig. 2a, asterisks). This striking reduction of H4K16ac could result from decreased levels of MOF in *MSL3* patient cells. However, MOF immunostaining displayed a homogenous distribution within all cells of the tissue section (Fig. 2b). This indicates that the loss of H4K16ac is not caused by the reduction in MOF levels per se. Rather, it is due to a misregulation of its enzymatic activity.

We confirmed these observations in primary fibroblast cells established from skin punches, where cells lacking functional MSL3 also displayed a severe decrease in H4K16ac (Fig. 2c,d), but not in other histone modifications tested (for example, H3K27me3; Fig. 2e and Supplementary Fig. 2b). Remarkably, quantification of these stainings showed a global reduction of H4K16ac in these heterozygous $X^{MSL3-WT}/X^{MSL3-MUT}$ female patient samples (Fig. 2d). Such a staining pattern is unusual for an X-linked gene in females, where conventionally, heterozygous mutations would be expected to result in a 50:50 population of wild-type and mutant cells. Targeted sequencing of *MSL3* complementary DNA (cDNA) for patients 1 and 2 demonstrated mild co-expression of the mutated and wild-type allele at the RNA level (Supplementary Fig. 2d). While the nature of *MSL3* mutation in P14 (whole gene deletion) precludes the expression of any mutant MSL3 protein, we note that P1/2 proteins were highly unstable (Fig. 1h), making it unlikely that the *MSL3*-linked syndrome is generally caused by a dominant negative effect. However, human *MSL3* was recently found to be a facultative escapee²² that is stochastically and variably expressed from both female X chromosomes. We therefore propose that *MSL3* haploinsufficiency might cause the variable but consistent reduction of H4K16ac in female patients.

Since there could be potential crosstalk between H4K16ac and other residues on the histone tails²³, and given that MSL3 has been linked to H3K36me3 and H4K20me1^{24,25}, we validated the above findings by bulk western blot analyses and quantitative liquid chromatography–mass spectrometry (LC-MS). In line with the immunofluorescence, we exclusively scored a reduction of MSL3 and H4K16ac levels, while MOF and other histone modifications remained unaffected in western blots (Fig. 2f, and Supplementary Figs. 2b and 5). Affirming these results, when comparing the three patients with two different controls by comprehensive histone LC-MS analysis, the modification that was by far the most significantly affected—both by *P* value and fold-change—was H4K16ac (Fig. 2g, Supplementary Fig. 2e,f, and Supplementary Table 2). Hence, loss of MSL3 in patients appears to primarily affect H4K16ac.

H4K16ac has previously been linked to cell-cycle regulation^{21,26–28}. We noticed that patient-derived fibroblasts grew significantly slower than Control cells (Supplementary Fig. 2g). Nevertheless, no clear difference of any specific cell-cycle phase could be scored (Supplementary Fig. 2h). Cells did not show signs of G1/S arrest either by reverse transcription quantitative PCR (RT-qPCR) for senescence markers, or by X-gal staining (Supplementary Fig. 2i,j). Furthermore, no differences were scored for γ H2AX signal, indicating no apparent increase in DNA damage (data not shown).

To further dissect the possible cause for the slow growth of female *MSL3* mutant cells, we next challenged them by the removal of serum and tested their capacity to respond to changes in environmental conditions. Interestingly, on performing these starvation experiments, we observed that patient-derived fibroblasts showed a delay in exiting the G0 phase (Supplementary Fig. 2k). This suggests that *MSL3* mutant cells may fail to timely respond to growth factor signals—a property that is crucial during development.

Transcriptome deregulation in cells from patients with *MSL3* mutations. Since MSL3 is part of a chromatin-modifying complex involved in transcription regulation^{21,29,30}, we next performed RNA sequencing (RNA-Seq) experiments on three patient-derived primary fibroblast cell lines. For this purpose, we analyzed two different passages compared with a passage-matched Control in synchronized conditions. Differential expression analysis was performed using DESeq2, and genes with an FDR < 0.05 were considered differentially expressed (Fig. 3a). As gene expression differences can reflect genetic background or environmental influences between individuals³¹, we decided to consider only transcripts that were consistently deregulated in all three patients. This identified a set of 519 commonly misregulated genes (Fig. 3a,b and Supplementary Fig. 3a).

We first explored whether altered expression of these genes could be directly related to MSL-mediated H4K16ac. We noticed that the extent of misregulation (\log_2 [fold-change]) was much more consistent between different patients in the downregulated subset compared with the upregulated group of genes (Fig. 3a,c). This supports the idea that the upregulated group of genes arise from secondary responses within each individual, which may be more variable. We also found that the orthologs of the differentially expressed genes in patient cells were significantly enriched for mMSL2 chromatin immunoprecipitation (ChIP) peaks in mouse cells²¹. In contrast, mKANSL3 peaks were significantly under-represented, corroborating the hypothesis that the observed effects are caused by H4K16ac deposited by the MSL complex, but not the KANSL complex (Fig. 3a,d). Therefore, we performed H4K16ac ChIP experiments in patient cells and focused on conserved mammalian MSL targets that are mMSL2 bound in mouse cells and very consistently downregulated in both patient cells, as well as on *MSL3* knockdown in male and female fibroblasts (Fig. 3e). These results substantiated our conclusion, as H4K16ac was lost from *ZNF185* and *SPON2*, but not *H3F3B* (Fig. 3e and Supplementary Fig. 3b).

We next focused on analyzing the biological functions and pathways orchestrated by the differentially expressed genes in the *MSL3*-linked syndrome. Intriguingly, we found differentially expressed Notch, Hox and Wnt family members, which are key morphogenetic regulators of development from *Drosophila* to mammals. We confirmed these findings by RT-qPCR, for which we included several additional control cell lines (Control 2 and Control 3). Remarkably, patient cell lines showed very consistent behavior in the selected set of downregulated as well as unaffected control genes (Fig. 3f, Supplementary Fig. 3c, and Supplementary Table 5). We could also confirm the reduction at the protein level for HTR7 (5-hydroxytryptamine receptor 7) and UNC5B (netrin receptor UNC5B) in the patient skin specimens (Supplementary Fig. 3d).

Pathway perturbation analysis highlighted ‘dermatological diseases’, ‘organismal abnormalities’, ‘gastrointestinal disease’, and ‘neurological disease’ as top-scoring disease signatures (Fig. 3g). More specifically, the genes commonly affected on *MSL3* mutation are overall involved in the activation of cellular movement, organization of the cytoskeleton, or the formation of cell protrusions, while mechanisms to reduce cell death are repressed (Fig. 3h). These are all characteristic symptoms of the *MSL3*-linked syndrome underscoring the relevance of our RNA-Seq dataset (see Supplementary Table 3 for differentially expressed and clinically relevant genes based on patient phenotypes).

Next, we were interested in dissecting the primary gene expression perturbations caused by acute loss of *MSL3* versus secondary effects that may accumulate in patients during development. Therefore, we performed *MSL3* knockdown experiments in control primary fibroblast cell lines, as well as *MSL3* rescue experiments in patient cells, to determine the extent to which we could phenocopy the gene expression changes detected by RNA-Seq. We observed an acute downregulation of multiple target

genes (for example, *ZNF185*, *SPON2*, *NOTCH3*, and *FAM157*) on *MSL3* knockdown in both male and female cells (Supplementary Fig. 3e). Genes found to be upregulated in patient cells typically did not respond (for example, *PRICKLE*) or were sometimes downregulated (*WNT2*). When we performed the inverse experiment by providing *MSL3* in patient cells, we noted a similar trend (Fig. 4a). We could rescue several downregulated targets (*ZNF185*, *SPON2*, *FOSB*, and *EGR2*) by re-expressing *MSL3*. Interestingly, these genes were also upregulated in Control cells, indicating that they might be more generally susceptible to alteration of *MSL3* and H4K16ac levels. Taken together, our gene expression analysis suggests that a lack of the MSL complex results in acute downregulation of several biologically relevant genes, including key developmental regulators. This misregulation may then gradually trigger secondary perturbances, collectively resulting in the global disease state of the *MSL3* syndrome.

Histone deacetylase inhibitor (HDACi)-mediated alleviation of phenotypes in patient cells. Given the clear link between H4K16ac and *MSL3* mutation, we considered whether it would be possible to rescue the phenotypes of patient cells not only by reintroducing *MSL3* (Fig. 4a) but also by readjusting acetylation levels. We therefore tested three different classes of HDACi that are currently approved for clinical trial phase II or III (SAHA (suberoylanilide hydroxamic acid; also known as vorinostat)^{32,33}, LBH-589 (panobinostat)³⁴, and MGCD0103 (mocetinostat)³⁵), as well as trichostatin A as a positive control^{36,37}. These inhibitors show strong effects on histone H4 acetylation and, in particular, H4K16ac in human transformed cells³⁷ (Supplementary Fig. 4a). Hence, we referred to the aforementioned study for the use of HDACi to modulate H4K16ac in our primary untransformed cells.

Treatment of Control and patient cells with HDACi resulted in bulk histone hyperacetylation and triggered upregulation of acetylation-sensitive genes (Supplementary Fig. 4b–d). We first verified that the response to HDACi is effective in all mutant cells, irrespective of the *MSL3*/H4K16ac levels. We therefore performed immunostainings on HDACi-treated patient cells and observed an overall increase of H4K16ac at the single-cell level from both high and low *MSL3* populations (Fig. 4b). Quantification of these stainings in patient-derived cells confirmed the rescue of H4K16ac, which was now comparable to the healthy Control (Fig. 4c). Next, we tested whether this has a positive influence on gene expression. Remarkably, treatment with HDACi specifically rescued the expression level of selected downregulated targets, while control genes or upregulated targets remained largely unchanged (Supplementary Fig. 4e; other data not shown). Unlike P1, P2 and P14 patient cells responded more selectively to LBH-589, while the other two HDACi (SAHA and MGCD0103) showed only a modest rescue of gene expression defects (Supplementary Fig. 4e). To characterize the HDACi response in *MSL3*-deficient cells in a more comprehensive manner, we performed RNA-Seq on treatment with LBH-589. As expected, HDACi treatment caused major transcriptional changes (Supplementary Fig. 4f and Supplementary Table 4). However, we successfully observed previously detected pathways, as well as potential side effects of this drug in Control and patient cells³⁸ (Fig. 4d and Supplementary Table 4). This supports the notion that the primary HDF cell lines used in our analysis represent a relevant model for studying the *MSL3*-linked syndrome.

Interestingly, a large set of the differentially expressed genes reversed their expression toward normal levels on HDACi treatment, although the extent varied between different genes and patients (Supplementary Fig. 4g and Supplementary Table 3). We focused our attention on the 116 genes that were fully rescued to control levels and hence did not score as differentially expressed after panobinostat treatment (Fig. 4e). Among them, clinically relevant genes based on the patient phenotypes were scrutinized. One

would expect a positive clinical impact by restoring a basal expression of, for example, *SOX11* (OMIM 615866), *HOXD10* (OMIM 142984), *LEP* (OMIM 164160) or *ACTG2* (OMIM 102545) based on their role in human disease and patient symptoms, including cognitive performance, gait disturbance, being overweight, and feeding difficulties/constipation, respectively. Accordingly, KEGG pathway analysis indicated that HDACi may generally rewire deregulated processes relevant to the disease state of these patients (Fig. 4f and Supplementary Table 3).

Hence, we wondered whether we would be able to rescue the previously observed cell-cycle phenotype and migratory signature using HDACi. Indeed, genes involved in G0 arrest (for example, *GAS1*), were rescued toward the expression levels in Control cells on HDACi treatment (Supplementary Fig. 4h), while displaying a slight increase in G1/S arrest. Moreover, in agreement with the activation of the respective cellular pathways (Fig. 3h), we had observed that *MSL3* patient-derived fibroblasts migrate faster than Control cells. Ergo, we assessed whether the positive influence of HDACi on gene expression would also lead to a beneficial outcome on a cellular level. Strikingly, on HDACi treatment, we could restore the phenotype of *MSL3* mutant cells, as they now adopted the migratory behavior of the Control cells (Fig. 4g,h and Supplementary Fig. 4i). Therefore, HDACi have the potential to both molecularly as well phenotypically alleviate at least some of the defects in *MSL3* mutant cells, thus providing a promising therapeutic avenue for these patients.

Discussion

In summary, we report a syndrome where loss of *MSL3* is associated with a pronounced loss of bulk H4K16ac. The patients presented in our study display a complex phenotype including neurodevelopmental delay, with a recurrent facial dysmorphism and a progressive neurological disorder. This suggests that MSL-mediated H4K16ac in humans may be involved in the regulation of complex developmental processes; for example, those occurring in the brain^{5,39,40}. This is intriguing as it is in contrast with the sex-specific function of *MSL3* during dosage compensation in flies, suggesting a more context-specific role in mammals. Given the conserved role of H4K16ac in chromatin decompaction from yeast to humans, it remains to be demonstrated why its physiological roles evolved to be so organism specific. Our study also raises a general question about the role of H4K16ac, as *MSL3* patients survive to adulthood compared with the early embryonic lethality of the *Mof* mutant in mice^{41–44}. An important point to consider in this context is that although *MSL3* mutations lead to a severe loss of H4K16 acetylation, remaining H4K16ac may be sufficient to allow developmental progress to a certain extent, since *MOF* is still expressed in these cells. While no *MOF* variants have been reported in any developmental disorder yet, de novo heterozygous loss-of-function variants of *KANSL1* were reported to cause Koolen–de Vries syndrome (KdVS)^{45–47}. *KANSL1* encodes a scaffold component of the MOF–KANSL complex. However, the disease characteristics of KdVS are symptomatically and molecularly distinct from patients with *MSL3* mutations with regard to features of facial dysmorphism—specifically, palpebral fissure orientation, nose tip and mouth shape, and visceral and brain abnormalities, which are absent in patients with *MSL3* mutations. Although KdVS patients also display neuromuscular disorders, the progressive gait disturbance observed in the *MSL3*-linked syndrome may be a distinctive feature. Given the multifaceted and widespread roles of MOF in processes such as metabolism⁴⁴, autophagy⁴⁸, and stress response⁴³, we speculate that the *MSL3* mutations presented here may provide separation-of-function alleles, allowing us to dissect the role of altered H4K16ac deposited by the MOF–MSL complex versus the MOF–KANSL complex during human development.

The *MSL3*-linked syndrome affects both hemizygous males and heterozygous females with comparable symptoms, which is difficult to reconcile with classical models of X-chromosomal inheritance⁴⁹. More recently, it has been appreciated that X-linked disorders can display intermediate penetrance; for example, due to varying degrees of X chromosome inactivation in females⁵⁰. This includes variations in escape genes, such as *IQSEC2*⁵¹, or examples like *XLH* or *E1a*, where similar phenotypes can be observed in both heterozygous females and hemizygous males. For *MSL3*, this can be presumably linked to its property of being a facultative escapee, where various degrees of X chromosome inactivation and escape might provide an explanation for the phenotypic and molecular differences among female patients. This is notable for the mother of P16, who is a carrier of the disease-causing allele, but is only mildly affected. The nature of the *MSL3* variants, as well as environmental effects modulating epigenome plasticity, might additionally contribute to the phenotypic outcome of the syndrome. A comprehensive assessment of the allele-specific expression of *MSL3* in different types of tissues, and the establishment of alternative cellular and animal models, will be required to dissect the apparently very complex regulation and phenotypic outcome in the *MSL3*-linked syndrome in both sexes.

Lastly, the positive outcome of HDACi partially alleviating migratory and cell-cycle defects provides a preliminary, yet promising ground for future studies, especially in light of the degenerative nature of the *MSL3*-linked syndrome. More efforts will need to be undertaken to potentially ameliorate the symptomatology and delay the probable myelopathy-caused hyperreflexia, for which HDACi have already shown positive results⁵². The possibility of alleviating at least some of the symptoms of these patients through repurposing of anticancer drugs provides yet another case where epigenetic therapy using HDACi may pave ways for treatments of other diseases affecting epigenetic modifiers. Taken together, our characterization of this syndrome not only provides insights into the developmental roles of MSL-associated H4K16ac in humans, but also underscores the general relevance of studying sex-specific aspects related to X-linked diseases.

URLs. National Heart, Lung, and Blood Institute Exome Sequencing Project Exome Variant Server, <http://evs.gs.washington.edu/EVS/>; Database of Single Nucleotide Polymorphisms (dbSNP), <http://www.ncbi.nlm.nih.gov/projects/SNP/>; Picard, www.picard.sourceforge.net; University of Burgundy Centre de Calcul, <https://haydn2005.u-bourgogne.fr/dsi-ccub/>; SeattleSeq Annotation tool, snpgs.washington.edu/SeattleSeqAnnotation137/; ExAC Browser, <http://exac.broadinstitute.org/>; Genome Analysis Toolkit (GATK) HaplotypeCaller, https://software.broadinstitute.org/gatk/documentation/tooldocs/3.8-0/org_broadinstitute_gatk_tools_walkers_haplotypecaller_HaplotypeCaller.php; European Bioinformatics Institute (EMBL-EBI) Expression Atlas, <https://www.ebi.ac.uk/gxa/home/>; Ensembl Biomart, <https://www.ensembl.org/biomart>; Integrative Genomics Viewer, <https://software.broadinstitute.org/software/igv/>; DECIPHER, <http://decipher.sanger.ac.uk>; ProteomeXchange Consortium, <http://proteomecentral.proteomexchange.org>; National Center for Biotechnology Information (NCBI) Gene Expression Omnibus, <https://www.ncbi.nlm.nih.gov/geo/>; NCBI ClinVar, <https://www.ncbi.nlm.nih.gov/clinvar/>.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at <https://doi.org/10.1038/s41588-018-0220-y>.

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Author contributions

M.F.B., A.A., and J.T. conceived the project and planned the experiments. The review of phenotypes and sample collection were performed by J.M., I.B., M.K., H.G.B., O.V., K.Ö., B.M., T.Y.T., S.V., K.L., A.R., D.S.J., A.F., V.M., R.B.F., V.M.K., A.-L.B., L.F., S.E.C., J.T., C.T.R., I.v.d.B., M.T.C., and M.W. Fibroblast isolation, tissue section preparation and processing, cell culture, protein analysis, FACS, immunostaining, microscopy, drug treatment experiments, ChIP, RNA expression analysis, and IPA pathway analyses were performed by M.F.B. A.-L.B. initiated data sharing and compiled patients' clinical data. G.S. performed deep-sequencing data analysis and manuscript editing. C.I.K.V. contributed to the design, analysis, and interpretation of the experiments and data. T.A. performed cloning of the MSL3 expression constructs and co-immunoprecipitations. W.G.S. and G.M. performed LC-MS sample preparation and analysis. T.R. purified recombinant proteins, and performed HAT assays and in silico modeling. Ethical consultation was provided by L.F. Data analysis for exome and frequency calculation was performed by Y.D., S.P., J.T., A.-L.B., C.G., and R.P. The manuscript was written and edited by C.I.K.V., M.F.B., A.A., and J.T. All authors reviewed, edited, and approved the paper.

Competing interests

The authors declare no competing interests.

Additional information

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Methods

Subjects, whole-exome sequencing, and variant validation. Sixteen individuals from 15 unrelated families with a disease-causing variant in *MSL3* were included in this study after we obtained informed consent. The local ethics committee of the Dijon University Hospital approved this study (2016-A01347-44). The patients were followed up by clinical geneticists from France, the United Kingdom, the Netherlands, Belgium, Denmark, Germany, the US, Estonia, and Australia. Thirteen variants were identified by whole-exome sequencing for the diagnosis of an undiagnosed rare disorder associated with multiple congenital anomalies and syndromic intellectual disability. Two deletions were identified by array comparative genomic hybridization and SNP array, and the X chromosome inversion breakpoint in *MSL3* was mapped by fluorescent in-situ hybridization and cloning of the junction fragment. The clinical observations were gathered through the Matchmaker Exchange initiative.

Blood or DNA samples from affected individuals and their parents, as well as informed consent, were obtained from all participants in accordance with the site-specific institutional boards of each country. Dedicated consent for the collection of skin biopsies was obtained.

De novo variants were analyzed by PCR and direct Sanger sequencing using DNA from patients and their parents. Primer sequences and detailed methods are available on request. In the family with two affected siblings (P5 and P6) and suspected parental somatic mosaicism, confirmation and estimation of the percentage of cells bearing the variant was performed using DNA extracted from the peripheral blood and saliva of all members of the family (the two patients and parents). Targeted sequencing for the variant position was performed on a MiSeq instrument (Illumina) at depths of 1,706 and 1,398 folds for the variant position in the father and mother, respectively. Genotyping identified 12 and 6 variant alleles, resulting in allelic balances of 0.007 and 0.004, respectively. In P1, the splice variant analysis was conducted on total RNA extracted from peripheral blood with TRIzol (Life Technologies). Sanger sequencing confirmed the splicing defect. Written consent was obtained from the parents and/or guardians of the affected individuals both for participation in the study and for use of the clinical photographs in this report.

Enrichment of de novo *MSL3* variants in the matchmaker cohort. The statistical significance after Benjamini–Hochberg correction for enrichment of functional and/or loss-of-function de novo mutations in the matchmaker cohort was estimated as previously described⁵³. The gene-specific mutation rates were published⁵⁴. Because *MSL3* is X linked, we assumed that the matchmaker cohort had an equivalent proportion of males and females (5,000 each; 15,000 alleles). The uncorrected *P* value was corrected for multiple testing, taking 18,280 genes to apply the Benjamini–Hochberg FDR correction.

Exome analysis. Each collaborating team followed a published methodology for sample and library preparation, sequencing data production, analysis, and interpretation^{55–59}.

Parental segregation analysis. Genomic DNA was amplified by PCR using a HotStarTaq PCR kit (Qiagen). PCR products were purified using an Agencourt CleanSEQ system (Beckman Coulter) and sequenced with a BigDye Terminator Cycle Sequencing kit (version 3.1; Applied Biosystems) on an ABI 3730 sequencer (Applied Biosystems). Sequence data were analyzed using Mutation Surveyor version 4.0.9 (SoftGenetics).

MiSeq cDNA analysis. Total RNA was extracted from peripheral blood with TRIzol (Life Technologies). The integrity of the RNA was assessed on 1.2% agarose gel, and the concentration and purity were determined by optical densitometry. cDNA was generated with a QuantiTect Reverse Transcription kit (Qiagen) and PCR amplified (Supplementary Table 5) with PrimeStar GXL (Takara). A Nextera XT DNA Prep Kit (Illumina) was used to create a cDNA library and sequenced on a MiSeq instrument (Illumina). Resulting data were aligned to the human reference genome (GRCh37/hg19) using STAR2 (version 2.5.3)⁶⁰. Aligned reads were sorted using Picard (version 2.17). Splice junctions and transcript organization were visualized using the sashimi plot of the Integrative Genomics Viewer.

Haplotype. The *MSL3* locus was amplified from genomic DNA by long-range PCR using PrimeSTAR GXL (Takara). PCR products were pooled for each case. The Nextera XT DNA Prep Kit (Illumina) was used to create a tagged and fragmented DNA library, which was sequenced on the MiSeq (Illumina). Resulting data were aligned to the reference genome (GRCh37/hg19) using the Burrows–Wheeler Aligner (version 0.7.6). GATK (version 2.1-10) enabled indel realignment and base quality score recalibration. Variants with a quality score of >30 and an alignment quality score of >20 were annotated with SeattleSeq Annotation (version 137). The haplotype was determined using HaplotypeCaller from VCF files.

Protein modeling. The complex structure of the MSL1–MSL3 complex (Protein Data Bank: 2Y0N) was analyzed and visualized with Pymol (version 1.7.4)

Cloning for protein expression and purification. The human MOF_{174–458} and MOF_{2–458} (UniProt Q9H7Z6-1) CDS were cloned in a modified pET15b vector

(His₁₀-tag). MSL1_{470–520} or MSL1_{471–616} (UniProt Q68DK7-1) CDS were cloned into the multiple cloning site (MCS) 1 of a pACYC coexpression vector (Novagen) with MOF_{174–458} cloned in MCS2. The coding sequence of MSL3_{167–517} (UniProt Q8N5Y2-1) was cloned in MCS1 of a modified pRSFduet vector. Proteins were expressed in lysogeny broth (LB) using Rosetta2 pLysS (MOF_{174–458} and MOF_{2–458}) or BL21(DE3) cells (MOF_{174–458}–MSL1_{470–520} and MOF_{174–458}–MSL1_{471–616}–MSL3_{167–517}). Expression was induced with isopropyl β-D-1-thiogalactopyranoside (0.1–1 mM, OD₆₀₀ 0.5–0.7, 18 °C overnight). After lysis by sonication (MOF_{174–458}, MOF_{174–458}–MSL1_{470–520}, and MOF_{174–458}–MSL1_{471–616}–MSL3_{167–517}; 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 500 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol, 0.01% (w/v) taurodeoxycholate, 1 ethylenediaminetetraacetic acid (EDTA)-free cOmplete (Roche), pH 7.5; MOF_{2–458}; 50 mM HEPES, 100 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol, 0.01% (w/v) taurodeoxycholate, 1 cOmplete (Roche), pH 7.5) and clarification by centrifugation, the supernatant was applied to a HisTrap HP column (GE Healthcare). This was washed with lysis buffer (for MOF_{174–458} and MOF_{174–458}–MSL1_{471–616}–MSL3_{167–517} containing 2,000 mM NaCl) before elution with an imidazole gradient (20–300 mM). Protein-containing fractions were pooled.

After buffer exchange (50 mM HEPES, 100 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol, 0.01% (w/v) taurodeoxycholate, pH 7.5), the solutions containing MOF_{2–458} and the trimeric MOF complex were applied to a Heparin HP column (GE Healthcare) and eluted with a NaCl gradient (100–2,000 mM NaCl). Protein-containing fractions were pooled and concentrated. Finally, MOF_{174–458}, MOF_{2–458}, MOF_{174–458}–MSL1_{470–520}, and MOF_{174–458}–MSL1_{471–616}–MSL3_{167–517} were applied to a Superdex S75 (MOF_{174–458}, MOF_{2–458} and MOF_{174–458}–MSL1_{470–520}) or S200 (MOF_{174–458}–MSL1_{471–616}–MSL3_{167–517}) gel filtration column (25 mM HEPES, 500 mM NaCl, pH 7.5). Protein-containing fractions were pooled, concentrated, flash-frozen, and stored at –80 °C. Chromatography was performed on Äkta purifier systems. The purity of all assayed proteins and complexes was >90%. The protein concentration was determined by Bradford assay.

Nucleosome reconstitution and HAT assays. Nucleosomal core particles were reconstituted using a PCR-amplified 147-base pair DNA fragment⁶¹ with 5' Cys5 and 3' Biotin and *Xenopus laevis* histone octamers applying salt dialysis⁶². The nucleosomal core particle quality was assessed using agarose gel electrophoresis. For HAT assays, 100 nM MOF_{174–458}, MOF_{2–458}, MOF_{174–458}–MSL1_{470–520}, or MOF_{174–458}–MSL1_{471–616}–MSL3_{167–517} were mixed with AcCoA (Sigma; final concentration 33 μM) and nucleosomes (0.54 μg) in assay buffer (10 mM Tris, 200 mM NaCl, 1 mM EDTA, pH 8.0), and incubated for 1 h (37 °C, 600 r.p.m.). The reaction was stopped by the addition of a 4× sodium dodecyl sulfate (SDS) sample buffer followed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot.

Cell culture, drug treatment, and transfections. Primary HDFs were isolated from 3 mm skin biopsies using 0.25% collagenase type I, 0.05% DNase I, 20% fetal calf serum high glucose Dulbecco's modified Eagle's medium (DMEM; Gibco) overnight at 37 °C. HDFs were allowed to attach and cultured in DMEM supplemented with 10% fetal calf serum, penicillin-streptomycin. All experiments were performed at early passages (2–8). LBH-589 (Selleck Chemicals), MGCD0103 (Toronto Research Chemicals), SAHA (Adipogen) and trichostatin A were dissolved in dimethyl sulfoxide (DMSO). For most experiments, before experimental treatment, cells were starved overnight in Hank's Balanced Salt Solution (24020133; Thermo Fisher). Flp-In T-Rex 293 cells (R78007; Thermo Fisher) were maintained in DMEM-GlutaMAX supplemented with sodium pyruvate, glucose and 10% fetal bovine serum. They were also maintained in zeocin- and blasticidin-containing medium according to the manufacturer's protocol. All transgenes used in immunoprecipitation experiments were cloned into pcDNA5/FRT/TO (V6520-20; Thermo Fisher) with a C-terminal 3×FLAG–HBH-tag (HBH, His6-biotinylation sequence-His6). Entire tag sequence in aa: LEDYKD HDGDYKDHDIYKDDDDDKRGSHHHHHHAGKAGEGEIAPALGATVSKILVKE GDTVKAGQTVLVLEAMKMETEINAPT DGKVEKVLVKERDAVQGGQLKIG VHHHHHH. The *MSL1* coding sequence was cloned with a C-terminal 3×HA-Avi tag. This vector was co-transfected with the *MSL3* wild type (full length), truncating mutants or a green fluorescent protein (GFP) coding sequence (as an immunoprecipitation negative control) in a 1:1 ratio (500 ng each on 6-well plates for nuclear FLAG-tag immunoprecipitation) with a 3:1 lipid (Lipofectamine 2000 (μl) to DNA (μg) ratio). Some 6 h after transfection, transgenes were induced with 0.1 μg ml⁻¹ doxycycline for 16 h before lysate preparation. The hsMSL3–3×FLAG–HBH coding sequence was subcloned into a custom pCAG–MCS–BGH (polyA), pSV40–BlasticidinR–SV40 (polyA), pSV40–mCherry–SV40 (polyA) vector created in multiple steps from an SV40 promoter containing backbone pMB1610 (Addgene 65853). The vector and map can be provided on request. For HDF transfections, 0.1 μg plasmid was used with Lipofectamine 3000 on 1 × 10⁶ cells in Opti-MEM followed by selection with 30 μg ml⁻¹ blasticidin-HCl (Invitrogen; A111139-03) overnight. Cells were then grown in fresh media for 24 h before RNA extraction.

FLAG immunoprecipitation. Nuclei were isolated with 0.5% NP-40 in isotonic lysis buffer (10 mM Tris HCl, pH 7.5, with 2 mM MgCl₂, 3 mM CaCl₂, and 0.3 M sucrose). Nuclear pellet was extracted using HMGT buffer (25 mM HEPES pH 7.5,

10 mM MgCl₂, 10% glycerol, 0.2% Tween-20) containing 420 mM KCl. The lysate was clarified by centrifugation before slowly reducing KCl to 150 mM by dilution. FLAG-M2 beads (Sigma; 1804) were used for overnight immunoprecipitation followed by HMG buffer (150 mM KCl) washes. Proteins were eluted by incubating for 10 min at 70°C in 2× SDS sample buffer.

MSL3 knockdown in HDFs. Silencer Select (Ambion–Thermo Fisher) small interfering RNA ID s21530 and control small interfering RNA were used at a final concentration of 50 nM for 32 h before RNA extraction.

Expression analysis in HDFs and RT–qPCR. Total RNA was prepared from 12-well-plate-cultured cells using TRIzol (Thermo Fisher) and purified with Direct-zol RNA MiniPrep and MicroPrep kits (Zymo Research). cDNA samples were synthesized using Maxima Reverse Transcriptase (K1671; Thermo Fisher) from 20–50 ng of total RNA. RT–qPCR reactions were performed on a LightCycler 480 Multiwell Plate 384 (Roche) using SYBR Green Master Mix (Roche). The primer efficiency was tested using serial dilutions.

Immunofluorescence and immunohistochemistry. HDFs were seeded as required for the experimental setup and fixed in 4% formaldehyde in a μ -Slide 8-Well on ice. Formalin-fixed, paraffin-embedded (FFPE) section and HDF immunostainings were performed according to published procedures⁶³ using MSL3 antibody (USB129933; MaxPab; 1:200) as primary, together with H4K16ac (07-329; Millipore; 1:500), H3K27me3 ((C36B11) 9733; Cell Signaling Technology; 1:250), E-cadherin (610181; BD Bioscience; 1:200), and KAT8 (EPRI5803) (ab200660; Abcam; 1:500) antibodies.

Multi-stacked and tiled images were acquired on a Spinning disk confocal microscope (Zeiss). The fluorescence intensity was measured on orthogonal projections from the same stack number using ImageJ (1.47v) and corrected for background. Fluorescently labeled secondary antibodies (Invitrogen) were used at a 1:300 dilution: Goat-anti-Mouse-Alexa Fluor 594 (A-11032), Goat-anti-Rabbit-Alexa Fluor 594 (A-11037), Goat-anti-Rabbit-Alexa Fluor 488 (A-11034), and Goat-anti-Mouse-Alexa Fluor 488 (A-11001).

Immunoblotting. Cells were fractionated using a subcellular fractionation kit (78840; Thermo Fisher), and the buffers were supplemented with cOmplete (Roche) and sodium butyrate (19-137; Merck Millipore). The protein concentration was determined using Qubit protein assay reagent. Samples were denatured at 95°C for 10 min in Pierce Lane Marker Reducing Sample Buffer, resolved by NuPAGE Bis-Tris denaturing conditions, and transferred to 0.22 μ m polyvinylidene difluoride membranes followed by blocking in 5% non-fat milk in phosphate buffered saline (PBS) (0.3% Tween) before incubation with antibodies: MSL3 (USB129933; MaxPab, USBiological, 1:1000 in bovine serum albumin), H3 (61475; Active Motif; 1:5000), H4K16ac (39167; Active Motif; 1:200 and 07-329; Millipore; 1:2000), Flag (M2; Sigma; 1:1000), HA (MMS-101P; Covance; 1:1000), MOF (A300-992A; Bethyl; 1:1000), DHX9 (NDH-II (E10) sc-137183; 1:2000–1:5000), H3K36me3 (ab9050; Abcam; 1:1000), acH3 (rabbit; Millipore; 1:1000), H4K20me1 (ab9051; Abcam; 1:1000), H4K8ac (61103; Active Motif; 1:1000), H3K14ac (39599; Active Motif; 1:1000), acH4 (06-598; Millipore; 1:1000), and H3K27me3 (9733; Cell Signaling Technology, 1:1000).

Mass spectrometry sample preparation. After histone extraction (78840; Thermo Fisher), proteins were separated on a 16% Tris-Glycine gel (Novex) and stained with colloidal Coomassie. Gel bands containing histones were cut out. After destaining, reduction, and in-gel alkylation, histones were propionylated in a 79:1 mixture of isopropanol and propionic anhydride (240311; Sigma) for 2 h. Gel pieces were washed with Milli-Q water, dried in a SpeedVac centrifuge and rehydrated in hydroxylamine followed by incubation with ammonium hydroxide solution to reestablish a neutral pH. After further washes with ammonium bicarbonate-based buffer and acetonitrile, gel pieces were again dehydrated. Subsequent tryptic digestion was performed as described previously⁶⁴. Recovered peptides were desalted on STAGE tips according to ref.⁶⁵.

LC-MS bottom-up analysis of histone peptides. Digested and desalted peptides were analyzed by tandem LC-MS (LC-MS/MS) using nanoflow high-performance liquid chromatography (Easy nLC1200; Thermo Fisher) coupled with a Q Exactive hybrid mass spectrometer (Thermo Fisher) through a nano electrospray ion source (Proxeon). Peptides were loaded on a 20-cm-long, 75- μ m-wide analytical column filled in-house with 1.9 μ m C18 beads (Reprosil C18; Dr. Maisch) at a flow rate of 500 nL min⁻¹. The analytical column was then equilibrated for 5 min (5% buffer A), followed by 60 min of peptide elution at a flow rate of 300 nL min⁻¹ with a linearly increasing concentration of organic solvent (80% acetonitrile, 0.1% formic acid). Q Exactive was operated in positive mode, employing data-dependent acquisition of fragmentation spectra of multiple-charged peptides for their identification.

MaxQuant software (version 1.6.0.16) was used for raw file peak extraction and protein identification. Peptide and protein FDRs were set to 0.01 using a decoy database. Additionally, ‘match between runs’ was used with a matching time window of 1 min and an alignment time window of 20 min.

LC-MS data analysis. A list of the ratios of the modified lysine or arginine sites derived from MaxQuant (‘...sites.txt’) was imported to Perseus⁶⁶. The data matrix was modified by applying the ‘expand site table’ step, removing empty rows, as well as contaminants and entries from the decoy database. All site intensities were log₂ transformed and normalized by subtracting the mean of the whole sample from the particular histone site intensities⁶⁷. A moderated *t*-test was used to test for significant differences between control and patient samples. Differences with a *q* value < 0.05 (Benjamini–Hochberg corrected) were considered significant.

Paraffin embedding and sectioning. Human skin specimens (2 mm) were fixed overnight in PBS containing 4% formaldehyde. After dehydration in ethanol/PBS and Histolemon series, they were incubated in paraffin at 58°C overnight and cast into molds covered with paraffin. Blocks were sectioned using an RM2155 microtome (Leica) at 5–10 μ m. Additional FFPE skin sections age matched to the control were purchased from BioCat.

Hematoxylin and eosin staining. Paraffin sections were dewaxed and rehydrated step-wise. The samples were stained in hematoxylin solution (0.1% hematoxylin, 5% KAl(SO₄)₂, 0.02% KIO₃). Counterstaining was performed by incubating the slides in eosin solution (1% eosin). Slides were mounted in Permount Mounting Medium (Thermo Fisher) and analyzed using brightfield microscopy.

Cell-cycle analysis by fluorescence-activated cell sorting (FACS). HDFs were synchronized by mitotic shake followed by serum starvation overnight. Cells were induced to cycle for 6 h by the addition of complete media. 1 × 10⁶ HDFs were fixed in suspension in 70% cold ethanol while gently shaking for 20 min at 4°C. After washing in FACS buffer (0.2% bovine serum albumin in PBS), H4K16ac staining was performed at room temperature for 90 min (39167; Active Motif; 1:500 and 07-329; Millipore; 1:500 in FACS buffer). This was followed by 30 min incubation with Ki67-FITC as indicated (BD Biosciences; 556026) and allophycocyanin-labeled Anti-Rabbit secondary antibodies. Propidium iodide (1 μ g ml⁻¹) was used to stain the nuclei. After additional washes, cells were analysed in FACS buffer using LSR II (BD Biosciences).

Alive singlets, based on forward scatter (FSC)/side scatter (SSC), were gated for further analysis. Three channel analyses were performed using antibodies/dyes with no fluorescence absorbance overlap. Machine laser intensities were set up using beads. For downstream analysis in FlowJo (version 10.4.1), alive cells were gated, followed by re-gating of only single cells. For cell-cycle phase analyses, the ‘cell cycle’ automatic function was run using propidium iodide.

Colorimetric detection of senescence-associated β -galactosidase. This was performed as described previously⁶⁸. Pictures were taken with a conventional charge-coupled device camera on a regular inverted cell culture microscope with a 10× objective.

RNA-Seq. Approximately 50 ng RNA was isolated from the synchronized control, P1, P2, and P14 HDFs at passage 4 and passage 7, and subjected to poly(A) RNA-Seq (TruSeq Stranded mRNA Sample Prep kit; Illumina). Reads were mapped to the GRCh37/hg19 reference genome with HISAT2 (version 2.2.0-beta)⁶⁹ using default parameters. Primary alignments were assigned to features with featureCounts (version 1.4.6-p2)⁷⁰ using the GRCh37 Gencode annotation (release 19). Differential expression analysis was performed using DESeq2 (version 1.18.1)⁷¹ with default settings. We identified differentially expressed genes between the control and all patients with patients treated as biological replicates at FDR-adjusted *P* < 0.05 (Control versus P1/P2/P14). When LBH-589-treated patients were compared with DMSO-treated controls (FDR < 0.05), we considered previously identified differentially expressed genes as ‘rescued’ if they were not differentially expressed anymore. To assess the global effect of LBH-589, genes were identified as differentially expressed in DMSO versus the LBH-589 treatment, irrespective of the cell line, at FDR-adjusted *P*_{adj} < 0.001. Network maps and pathways/cell components were generated in ingenuity pathway analysis (IPA; Qiagen; version 01-07) using curated lists from the Ingenuity Pathways Knowledge Base. Activation *z*-scores were used to infer the activation states (‘increased’ or ‘decreased’) of implicated biological functions. Differentially expressed genes were annotated using the OMIM morbid map (version November 2017).

ChIP sequencing (ChIP-Seq) analysis. MACS2 ChIP-Seq peaks of mMSL2, mMOF and mKANSL3³¹ were split in TSS (gene overlapping within \pm 1 kb of the TSS), gene body (gene overlapping from TSS + 1 kb until the gene end) or distal (closest gene to any peak) groups based on the NCBIM37 Gencode annotation (version 1) and compared with the differentially expressed genes in MSL3 patients. Mouse orthologues of differentially expressed MSL3 genes were identified on Ensembl BioMart. Overlaps, frequency calculations, statistical analyses, and plots were generated in R (version 1.1.383).

Scratch assay of HDFs. An equal number of cells were seeded in Culture Insert-2 (IBIDI) in 12 wells and allowed to attach. Cells were starved overnight before adding fresh media with LBH-589 (2 μ M) or DMSO. The cell-free gaps in which the cell migration could be visualized were created by removal of the silicon comb. Pictures

were taken with a conventional charge-coupled device camera on a regular inverted cell culture microscope with a 10× objective at 0, 6, 24, and 48 h after removal of the comb and drug administration. Images were analysed using ImageJ (1.47v); the cell-free gap was measured and plotted as ratio over time 0 for each corresponding cell line.

H4K16ac ChIP. HDFs were starved overnight and collected by centrifugation after cell counting. Cells were crosslinked using 1% formaldehyde for 10 min. Sodium butyrate was added to all buffers. Cells were fractionated (78840; Thermo Fisher) and nuclei dissolved in lysis buffer (20 mM Tris pH 8, 5 mM MgCl₂, 1 mM CaCl₂, 0.25 M sucrose, 1% Triton-X100, 10 μM EDTA, and 0.5% SDS). Some 50,000 lysed nuclei were diluted with 5× dilution buffer (10 mM Tris-HCl pH 8, 140 mM NaCl, 0.1% Sodium Deoxycholate, 1% Triton-X100, 1 mM EDTA, 1× protease, and 0.1% SDS) and chromatin sheared using a Diagenode Bioruptor Pico (7 cycles with 30 s ON/OFF Immunoprecipitation timing and antibody concentration were tightly set up using flow cytometry (50,000 nuclei, 2 h, room temperature, 1:750 dilution). Dynabeads-bound chromatin was washed using 2× immunoprecipitation dilution buffer, 1× High Salt wash buffer (400 mM NaCl), 1× LiCl buffer, and 1× TE (10 mM Tris, 1 mM EDTA, pH 8.0). Chromatin was reverse cross-linked at 65 °C overnight followed by RNase A and Proteinase K digestion. DNA was purified using phenol–chloroform extraction followed by ethanol precipitation.

Statistics. All statistics were calculated with GraphPad Prism 6/7 or R (version 1.1.383). One-way analysis of variance was performed for multiple comparisons followed by Bonferroni corrections for individual pairing. Dot plots are presented with the center line as the mean (average) ± s.e.m. In the box plots in Fig. 3c and Supplementary Fig. 2e, the line that divides the box into two parts represents the median, while the ends are the upper and lower quartiles (first and third quartile). The extreme lines shows the highest and lowest value excluding outliers. Bar plots represent the mean (average) ± s.e.m., with overlaid data points representing independent experiments. Results were considered significant at an FDR (*P* value) below 5%. Additional information and statistical analysis results are provided in the figure legends and Supplementary Table 5.

Reporting Summary. Further information on research design can be found in the Nature Research Reporting Summary linked to this article.

Data availability

RNA-Seq data have been deposited to the Gene Expression Omnibus under accession GSE102250. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org/>) via the PRIDE partner repository⁷² with dataset identifier PXD009317. The damaging variants reported as disease causing in this article were deposited in the ClinVar database under study accession SUB2871008.

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- Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated
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State explicitly what error bars represent (e.g. SD, SE, CI)

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Software and code

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Data collection

See "Data analysis" (next section)

Data analysis

Sequence data were analyzed using Mutation Surveyor v4.0.9 (Softgenomics). For MiSeq cDNA analysis, resulting data were aligned to the human reference genome (GRCh37/hg19) using STAR2 (v2.5.3)8. Aligned reads were sorted using Picard (v2.17). For Haplotype, Resulting data were aligned to the reference genome (GRCh37/hg19) using BWA (Burrows-Wheeler Aligner; v0.7.6). The Genome Analysis Toolkit (GATK; v2.1-10) enabled indel realignment and base quality score recalibration. Variants with a quality score >30 and alignment quality score >20 were annotated with SeattleSeq SNP Annotation (v137). Haplotype was determined using HaplotypeCaller (GATK). The complex structure of the MSL1-MSL3 complex (PDB: 2YON) was analyzed and visualized with Pymol (v1.7.4). Microscopy was analyzed using ImageJ (1.47v). For Mass Spectrometry, MaxQuant software (v1.6.0.16) was used for raw file peak extraction and protein identification. For downstream analysis of FACS, FlowJo (v 10.4.1) was used. For RNA-seq, reads were mapped to the GRCh37/hg19 reference genome with HISAT2 (v2.2.0-beta) using default parameters. Primary alignments were assigned to features using featureCounts (v1.4.6-p2) with the GRCh37 Gencode annotation (release 19). Differential expression (DE) analysis was performed using DESeq2 (v1.18.1) with default settings. Network maps and pathways/cell components were generated in Ingenuity Pathway Analysis (IPA, Qiagen Redwood City, Version 01-07 (01-07)). All statistics were calculated with GraphPad Prism 6/7 or R (v1.1.383).

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All RNA-seq data in this study have been deposited to the Gene Expression Omnibus under the accession number GSE102250.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository⁷⁰ with the dataset identifier with the dataset identifier PXD009317

The variants considered as disease-causing and reported in this article were submitted to Clinvar with the data-set identifier SUB2871008

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample size could not be predetermined as it was limited by the total number of only 16 human research participants / patients diagnosed with MSL3 syndrome participating in our study. Of those, 3 patients (p1, p2, p14) and one relative (ctrl1) agreed to undergo skin biopsies to derive fibroblast cultures. To match the number of patients and account for interindividual variability, two more control cultures were established (ctrl2, ctrl3). For most analyses, at least 3 independent experiments were performed whenever possible keeping in mind the sample limitations in our cell culture with these primary cells at early passages. For RNA-Seq, we chose a sample size of n=2 per genotype to keep with accepted practices in the field.
Data exclusions	No data was excluded.
Replication	To verify the reproducibility of the findings, we confirmed key findings of our study using several different and independent methodological approaches. For example, we independently verified the loss of H4K16ac in MSL3 syndrome using immunofluorescence, western blot and mass spectrometry. RNA-seq analyses were conducted by different researchers and same results were obtained. Further information on replication for each experiment are provided in the Figure Legends and methods. All attempts of replication were successful.
Randomization	Patients were allocated to the study groups by genotype (MSL3 wild-type versus MSL3 mutant). Adjusting for covariates was not possible in such a small study given the limited number of MSL3 patients and matched controls are not possible for the same reason.
Blinding	Blinding was not relevant, as there was no treatment of patients and all read-outs were objective.

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a	Involvement in the study
<input type="checkbox"/>	<input checked="" type="checkbox"/> Unique biological materials
<input type="checkbox"/>	<input checked="" type="checkbox"/> Antibodies
<input type="checkbox"/>	<input checked="" type="checkbox"/> Eukaryotic cell lines
<input checked="" type="checkbox"/>	<input type="checkbox"/> Palaeontology
<input checked="" type="checkbox"/>	<input type="checkbox"/> Animals and other organisms
<input type="checkbox"/>	<input checked="" type="checkbox"/> Human research participants

Methods

n/a	Involvement in the study
<input checked="" type="checkbox"/>	<input type="checkbox"/> ChIP-seq
<input type="checkbox"/>	<input checked="" type="checkbox"/> Flow cytometry
<input checked="" type="checkbox"/>	<input type="checkbox"/> MRI-based neuroimaging

Unique biological materials

Policy information about [availability of materials](#)

Obtaining unique materials Patient samples used in the study are restricted as they need consent from the patient families for further use.

Antibodies

Antibodies used

Immunofluorescence/FACS: anti-MSL3 antibody (USB129933, MaxPab, 1:200) as primary together with H4K16ac (07-329, Millipore, 1:500), H3K27me3 ((C36B11) 9733, Cell signalling, 1:250), E-cadherin (610181, BD Bioscience, 1:200), KAT8 [EPR15803] (ab200660, Abcam, 1:500). Ki67-FITC (BD Biosciences, 556026). Fluorescently-labeled secondary antibodies (Invitrogen) were used 1:300 dilution: goat-anti-mouse Alexa594 (A-11032), goat-anti-rabbit Alexa594 (A-11037), goat-anti-rabbit Alexa488 (A-11034), goat-anti-mouse-Alexa488 (A-11001).

Western Blots: MSL3 (USB129933, MaxPab, USBiologicals, 1:1000 in BSA), H3 (61475, Active Motif, 1:5000), H4K16ac (39167, Active Motif, 1:2000; 07-329, Millipore, 1:2000), Flag (M2, Sigma, 1:1000), HA (MMS-101P, Covance, 1:1000), MOF (A300-992A, Bethyl, 1:1000), DHX9 (NDH-II(E10) sc-137183, 1:2000-1:5000), H3K36me3 (ab9050, Abcam, 1:1000), acH3 (rabbit, Millipore, 1:1000), H4K20me1 (ab9051, Abcam, 1:1000), H4K8ac (61103, Active Motif, 1:1000), H3K14ac (39599, Active Motif, 1:1000), acH4 (06-598, Millipore, 1:1000), H3K27me3 (9733, Cell Signaling, 1:1000). Secondary HRP-labelled antibodies (1:10'000) used were anti-mouse IgG HRP (NXA931), anti-rat IgG HRP (NA935V) and anti-rabbit IgG HRP (NA934) from Sigma.

Validation

Antibodies used in this study were previously validated and published in multiple studies. Additionally, for each experiment we had relevant controls and independent experiments to ensure correct interpretation of results. Validation for results concerning histone modifications is provided by mass spectrometry (Figure 2g, Supplementary Figure 2f). MSL3 (C129933, MaxPab, USBiologicals, 1:1000 dilution): Western Blot for specificity is provided on <https://www.usbio.net/antibodies/129933/msl3l1-dkfpz586j1822-msl3-male-specific-lethal-3-homolog-male-specific-lethal-3-homolog-1-male-specific-lethal-3-protein-like-1>.

Our study further corroborates this, as MSL3 immunostaining and Western Blot signal is specifically and consistently affected in patients mutant for MSL3 but not healthy controls (Figures 2c, 2f, 4b). Specificity of the FLAG and HA-tag antibodies is provided in numerous studies and further corroborated in our study (e.g. Figure 1h). MOF antibody is validated in Chatterjee et al. (Cell. 2016 Oct 20;167(3):722-738.e23. doi: 10.1016/j.cell.2016.09.052., Figure S1D) and in: <http://www.abcam.com/kat8-myst1-mof-antibody-epr15803-ab200660.html>. DHX9 antibody validation: Nature. 2017 Apr 6;544(7648):115-119. doi: 10.1038/nature21715. E-Cad antibody specificity: <http://www.bdbiosciences.com/eu/applications/research/stem-cell-research/cancer-research/human/purified-mouse-anti-e-cadherin-36e-cadherin/p/610181>

Eukaryotic cell lines

Policy information about [cell lines](#)

Cell line source(s)

Cell line have been established as described in the method section. Patient samples used in the study are restricted as they need consent from the patient families for further use. FLPinTrex HEK293 cells were purchased from Thermo Fisher Scientific (R78007).

Authentication

Targeted MiSeq and RNA-seq confirms the nature of the established primary fibroblast lines. The cells were cultured in dedicated incubators, where no other human cell lines were grown. The Flp-In 293 T-REx cells were obtained from Thermo Fisher Scientific (R78007) and not further authenticated after purchasing.

Mycoplasma contamination

The cells were regularly tested for mycoplasma contamination by PCR (Jena Biosciences PP-401) and all tests were negative.

Commonly misidentified lines
(See [ICLAC](#) register)

No commonly misidentified cell lines were used in this study.

Human research participants

Policy information about [studies involving human research participants](#)

Population characteristics

Covariate-relevant population characteristics are provided in the Supplementary Table1. Adjusting for covariates was not possible in such a small study given the limited number of MSL3 patients and matched controls are not possible for the same reason. Information from Supplementary Table1:

Patient 1 Patient 2 Patient 3 Patient 4 Patient 5 Patient 6 Patient 7 Patient 8 Patient 9 Patient 10 Patient 11 Patient 12 Patient 13 Patient 14 Patient 15 Patient 16

Gender F F M M M M F F M M M F F M

Delivery term (weeks gestation) 36w 40w 36w 40w 38w 40 40w 38w Term 40w 39w 38w 39w 40w NA

Birth weight (centile) 15th 2nd NA NA NA NA 50th 75th NA 10th 25-50th 50th 25-50th NA 7th 97th

Birth length (centile) 16th NA NA NA NA NA NA 91th NA NA 25-50th 25-50th 50th NA 6th N

Birth OFC (centile) 98th NA NA NA NA NA NA 91th NA NA 50th 98th 50th NA 88th 97th

Growth parameters

Age at last follow up 4y 7y ½ 7y 2y 22y 24y 7y ½ 8y 16m 11.5 y 23y 13y 3y8m 13y 17y 4y

Weight (centile) 97th N N 45th NA NA 59th 91th 25-50th 86th 75-90th <1st 25th 50th 95th N

Length (centile) 24th N N 17th NA NA 9-25th 25th 0.4-2nd 57th 10-25th <3rd 25th 90-97th 12th N
 OFC (centile) >97th 50th N 97th NA NA 9-25th 75th 98-99th 96th >98th >98th 50th 50-75th 55th

Recruitment

The individuals screened in this study have been recruited through the Matchmaker Program with a diagnosis of likely damaging variants of MSL3. The affected individual and/or their family gave written consent for their inclusion in the analysis under a local ethic committees - approved protocol (2016-A01347-44).

Flow Cytometry

Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

HDFs were synchronized by mitotic shake followed by serum starvation overnight. Cells were induced to cycle for 6 hours by addition of complete media. 1'000'000 HDFs were fixed in suspension with 70% cold ethanol while gently shaking for 20 min at 4°C. After washing in FACS buffer (0.2% BSA in PBS), H4K16ac staining was performed at RT for 90min (39167, Active Motif, 1:500; 07-329, Millipore, 1:500 in FACS buffer). This was followed by a 30 min incubation with Ki67-FITC as indicated (BD Biosciences, 556026) and 2ndary anti-rabbit (APC labeled). Propidium iodide (PI) (1 µg/ml) was used to stain nuclei. Followed by additional washes, cells were analysed in FACS buffer using LSRII (BD, Heidelberg, Germany).

Instrument

LSRFortessa (LSRII)

Software

FlowJo (v 10.4.1)

Cell population abundance

In this study, cells were analyzed, but not sorted. The abundance / % of analyzed cells is presented in the figures.

Gating strategy

Single cells based on FSC/SSC were gated for further analysis. Example of the Gating Strategy is provided in Supplementary Figure 5. Three channel analyses were performed using antibodies/dyes with no fluorescence absorbance overlap. Machine Laser intensities were set up using beads.

- Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.