Breathing-in epigenetic change with vitamin C

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Vitamin C is an antioxidant that maintains the activity of iron and α-ketoglutarate-dependent dioxygenases. Despite these enzymes being implicated in a wide range of biological pathways, vitamin C is rarely included in common cell culture media. Recent studies show that reprogramming of pluripotent stem cells is enhanced when vitamin C is present, thereby illustrating previous limitations in reprogramming cultures. Here, we summarize understanding of dioxygenase function in reprogramming and epigenetic regulation. The available data suggest a link between dioxygenase function and stem cell differentiation, which is exposed to environmental influence and is relevant for human disease.

See the Glossary for abbreviations used in this article.

Introduction

Vitamin C also known as L-ascorbic acid or (5R)-[(1S)-1,2-dihydroxyethyl]-3,4-dihydroxyfuran-2(5H)-one, is an essential nutrient for humans and other primates. Most mammals synthesize ascorbate through a common metabolic pathway from glucose. However, primates, bats and guinea pigs have lost activity of the last, vitamin-C-specific enzyme, gulonolactone oxidase, in the pathway due to accumulation of mutations. Therefore, humans have to rely on nutrient sources including fruits and vegetables as a supply of vitamin C [1]. Vitamin C deficiency leads to a condition known as scurvy [2,3], which is characterized by a structural breakdown of the skin and epithelia due to loss of collagen hydroxylase function. In addition, vitamin C is involved in the synthesis of catecholamine, which acts as a neurotransmitter in the sympathic nervous system (Fig 1). Vitamin C is also required for the synthesis of carnitine, linking it to mitochondrial fatty acid transport [4]. Vitamin C has further been proposed to contribute to the regulation of several other pathways, including the hypoxia response, through regulating the activity of HIF prolyl-hydroxylases. One important aspect of vitamin C is that it acts as an electron donor for adjusting the redox state of iron-containing enzymes (Fig 2A,B). Vitamin C is by far the most effective compound for this reaction but is not absolutely essential. At least in some cases, vitamin C can be substituted by other antioxidants. Studies with vitamin-C-deficient mice have shown that glutathione can substitute fully for vitamin C in maintaining the activity of prolyl-hydroxylase function in the hypoxia response pathway [5]. Iron and α-ketoglutarate (2OG)-dependent dioxygenases are only active when iron is present in the Fe(II) state (Fig 2C). These enzymes catalyse oxidative decomposition of 2OG to carbon dioxide and succinate, thereby forming an Fe(IV)-oxo intermediate. The activated oxygen species can then be used to hydroxylate a specific substrate, which also restores the Fe(II) for the next catalytic cycle. In the absence of substrate, the iron atom is left in a higher oxidation state, which prevents further catalysis. Vitamin C acts as an electron donor for reducing iron whereby it is oxidized to dehydroascorbate (Fig 2B). Therefore, vitamin C performs a crucial function in maintaining the catalytic activity of Fe(II) 2OG-dependent dioxygenases.

For its general antioxidant properties, vitamin C is a frequent additive in food products and beverages. Its ubiquitous applications ensure a sufficient supply in the human diet. In the light of this, it seems surprising that ascorbate is rarely included in the formulations of cell culture media and supplements (Table 1). The awareness of vitamin C might have been historically dampened by the use of complex media components, such as sera that contain varying levels of vitamin C. Also the fact that common laboratory animals including rodents can synthesize vitamin C in their liver or kidney might have contributed to neglecting it in culture media. However, it needs to be considered that even for mammals that have an active gulonolactone oxidase (*Gulo*) gene, synthesis of ascorbate might not occur in cultured cells. Yet, attempts to demonstrate a beneficial effect of vitamin C in culture media have generally not been positive. This suggests that either standard cell cultures do not rely on vitamin-C-dependent pathways or that the lack of these pathways does not manifest itself in readily observable phenotypic consequences. Such realization of insufficient amounts of vitamin C comes from studies on reprogramming of induced pluripotent stem (iPS) cells. Addition of vitamin C enhances the generation of mouse and human iPS cells [6]. Furthermore, reprogramming in the presence of vitamin C results in iPS cells with an improved differentiation potential and maintains imprinted DNA methylation patterns on the Dlk1–Dio3 locus [7]. These results are surprising when considering the prevalent view that redox regulation is generally provided by addition of β-mercaptoethanol to culture media. Vitamin-C-dependent processes might have a crucial role in culture systems that involve reprogramming of cell types or change of cell fates. Examining the role of vitamin C in epigenetic regulation might be instructive and provide new ways of thinking.

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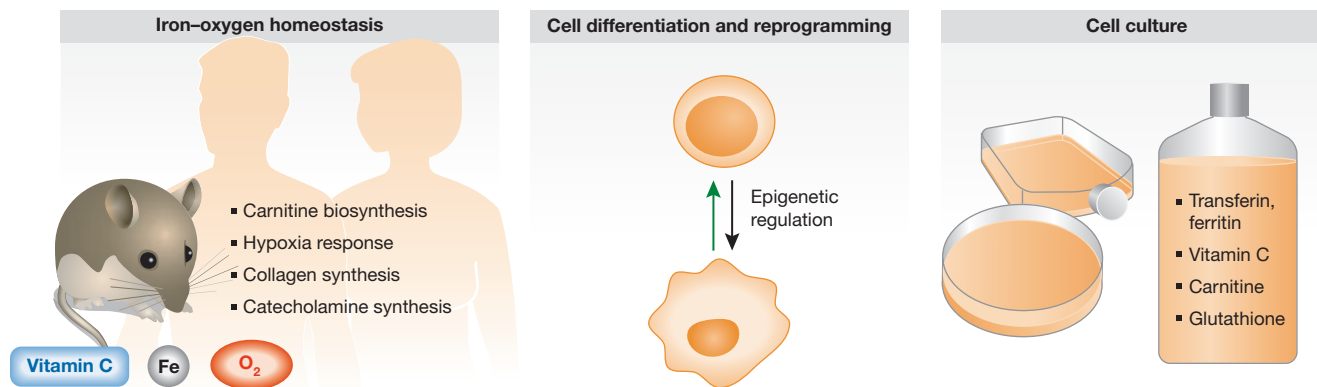
Fig 1 | Overview of processes influenced by vitamin C. Vitamin C regulates crucial enzymatic functions in the organism in cooperation with oxygen and iron metabolism. Dioxygenases are essential for regulating collagen, carnitin and catecholamine synthesis as well as the hypoxia response. In addition, dioxygenases catalyse chromatin, and DNA modifications thereby contribute to epigenetic regulation and cell differentiation. This aspect is also important in reprogramming of cell fates (green arrow). In the organism, iron (Fe) and vitamin C sources are environmental factors that affect the activity of dioxygenases. Similarly, in cell culture systems appropriate supplements can be used to mimic physiological levels of activity.

Glossary	
2OG	2-oxoglutarate
ABH	alkylation repair homologue
AlkB	alkylated DNA repair protein
Dio3	deiodinase, iodothyronine type III
Dlk1	delta-like 1 homologue
FTO	fat mass and obesity associated
HDAC2/4	histone deacetylase 2/4
HIF	hypoxia inducible factor
INK4/ARF	cyclin-dependent kinase inhibitor 2A
MEF	mouse embryonic fibroblast
MLL	myeloid/lymphoid/mixed-lineage leukaemia
Oct4	octamer-binding transcription factor 4

Dioxygenases contribute to epigenetic regulation

Vitamin C has a crucial role for the activity of the family of Fe(II)-2OG-dependent dioxygenases (Fig 2C). The catalytic domain is organized in a double-stranded β-helix (DSBH) fold, which consists of eight β-strands and contains a triad of two histidines and one carboxylate (aspartate or glutamate) that bind to Fe(II) [8]. This typical DSBH fold has been used to identify 2OG-Fe(II) dioxygenases from sequence information, and thereby link this catalytic mechanism with genes that have functions in regulating histone and DNA methylation [9,10].

JmjC-domain-containing histone demethylases. A class of Jumonji C (JmjC)-domain-containing enzymes function in the demethylation of lysine residues of histone proteins through an oxidative reaction that requires iron Fe(II) and 2OG (Table 2; [11–21]). Unlike the flavin cofactor-dependent histone demethylases, which only remove mono- and dimethyl-lysine modifications, some JmjC-domain-containing histone demethylases (JHDMs) can also demethylate trimethylated histone lysines [22–30]. The co-factor-bound JmjC domain is thought to produce a highly reactive oxoferryl intermediate that hydroxylates the histone lysine-methyl group, thereby generating an unstable chemical structure. Spontaneous elimination of formaldehyde completes the removal of the methyl group from the histone tail (Fig 2D). The JHDM group consists of many demethylases and can be divided further into several subfamilies based on phylogenetic

analysis [31]. Different members perform distinct demethylation reactions with varying specificity for the position of the lysine on the target histone and also for the methylation state. Their hydroxylase activity is mediated by the JmjC domain that contains two histidine residues and one glutamate residue, which chelate the catalytic iron atom and are invariably present in all family members, although not all proteins containing a JmjC domain are catalytically active. The JmjC domain is often present in association with additional domains that confer selective binding based on the methylation status of histones or DNA [32]. Depending on the size of the methyl-binding pocket, different members of the JHDM family are specific for mono-methyl-, dimethyl- or trimethyl-modified substrates [20]. In addition to lysine methylation, histone tails can also be mono- and dimethylated on arginine residues. Similarly to lysine methylation, arginine methylation influences chromatin remodelling and gene expression, and has been associated with cell fate transitions and plasticity regulation during development [33]. JHDMs might facilitate multiple functions in the adjustment or change of chromatin modification patterns. In addition, an important role in demethylating non-histone substrates is recognized but less completely understood.

TET proteins mediate oxidation of 5mC in DNA. Homology searches in mammalian genomes, for genes related to the hydroxylases involved in base J synthesis in trypanosomes, have led to the identification of the ten-eleven-translocation genes 1–3 (TET1–3), as members of a family of DNA hydroxylases that convert 5-methyl-cytosine (5mC) into 5-hydroxymethyl-cytosine (5hmC) (Fig 2E; [9,34]). Further conversion through iterative oxidation of 5mC results in the formation of 5-formylcytosine and 5-carboxyl-cytosine [9,34–36]. All TET proteins have a catalytic domain that contains a DSBH fold and binds to Fe(II) and 2OG. In addition, TET1 and TET3 but not TET2 have a zinc finger domain that might facilitate binding to or interaction with other proteins and DNA. 5hmC was found to be enriched at transcription start sites of bivalent gene promoters and exons in embryonic stem cells, indicating that TET enzymes contribute to the regulation of Polycomb group complex target genes [37,38]. This finding further suggests a potential role in the regulation of cellular identity [39–41]. Tet1 and Tet2 are expressed in mouse embryonic stem cells,

Fig 2 | Redox chemistry of vitamin C. (**A**) Ascorbic acid has chirality. L-ascorbic acid is vitamin C, whereas its enanziomere D-ascorbic acid does not confer the same biological effect. However, chemical antioxidant properties are equivalent in the L- and D-forms. (**B**) Oxidation of vitamin C to dehydroascorbic acid (DHA) yields two electrons that are used as a reducing agent in redox reactions. For the purpose of this review, these electrons are transferred to an iron atom to reduce its oxidation state. (**C**) Schematic overview of dioxygenase catalysis indicates different cycles of either substrate hydroxylation or vitamin-C-dependent regeneration. Molecular oxygen is used for the decarboxylation of 2OG, leading to the formation of an activated enzyme with an Fe(IV) intermediate (iron oxidation state is indicated in red). In the presence of substrate, the activated enzymatic complex hydroxylates the methyl group of the substrate and Fe(IV) is reduced to Fe(II) after the release of succinate. In the absence of a substrate, succinate is released without reduction of Fe(IV) leading to an inactive enzyme. Vitamin C can be used to reduce iron to Fe(II), restoring catalytic activity for the next cycle. Several histone- and DNA-modifying enzymes belong to the Fe(II) 2OG-dependent oxidase family. (**D**) Jumonji C (JmjC)-domain-containing histone demethylases catalyse the removal of methyl groups from lysine residues of histone proteins in a two-step mechanism. The first step, an hydroxylation reaction that converts the methyl group through an oxidative reaction that requires Fe(II) and 2OG, results in an instable intermediate. In a second step, spontaneous elimination of formaldehyde completes the demethylation reaction. (**E**) TET DNA hydroxylases catalyse the oxidation of methyl groups in the 5′-position of cytosine in DNA. Although the reaction seems to be similar overall to the first step in panel D, the hydroxymethyl group linked through a carbon–carbon bond is stable and is not spontaneously eliminated. 2OG, 2-oxoglutarate; TET, ten-eleven-translocation.

within which Tet3 expression is barely detected. The converse pattern is observed in fibroblasts, in which high expression of Tet3, intermediate expression of Tet2 and almost no expression of Tet1 is observed. It has further been shown that Tet genes are regulated by transcription factors associated with pluripotency. Depletion of Oct4 by RNA interference leads to the downregulation of Tet1 and upregulation of Tet3 with a concomitant decrease in the levels of 5hmC [39]. This indicates a crucial role for Tet1 in mouse embryonic stem cells. However, a targeted disruption of *Tet1* in mice is compatible with normal development [42], and a fraction of *Tet1*- and *Tet2*-double-mutants, despite presenting multiple developmental defects, can survive to adulthood [43]. These data suggest that Tet3 might also have overlapping functions with Tet1 and Tet2.

Mutations in *TET1* and *TET2* are also implicated in the development of several types of cancer and are associated with a decrease in 5hmC. *TET1* is an MLL partner in lymphoid and myeloid acute leukaemia, and mutations in *Tet2* are associated with myeloid leukaemias or myeloproliferative disorders, highlighting potential roles for TET enzymes in the regulation of cell identity [44]. Importantly, TET enzymes have been proposed as a crucial component of a DNA demethylation pathway [35]. Iterative oxidation of 5mC by Tet dioxygeneases results in the formation of 5-carboxylcytosine (5caC), which is recognized and excised by thymine DNA glycosylase. Tet1 has been implicated in DNA demethylation associated with genome-wide reprogramming during germline development in mice [45]. Tet3 has been shown to mediate hydroxylation of 5mC on the sperm-derived chromosomes in zygotes [46], whereas the maternally inherited chromosomes of the oocyte are protected from Tet3 activity by Stella binding [47]. This results in a differential modification of maternal chromosomes
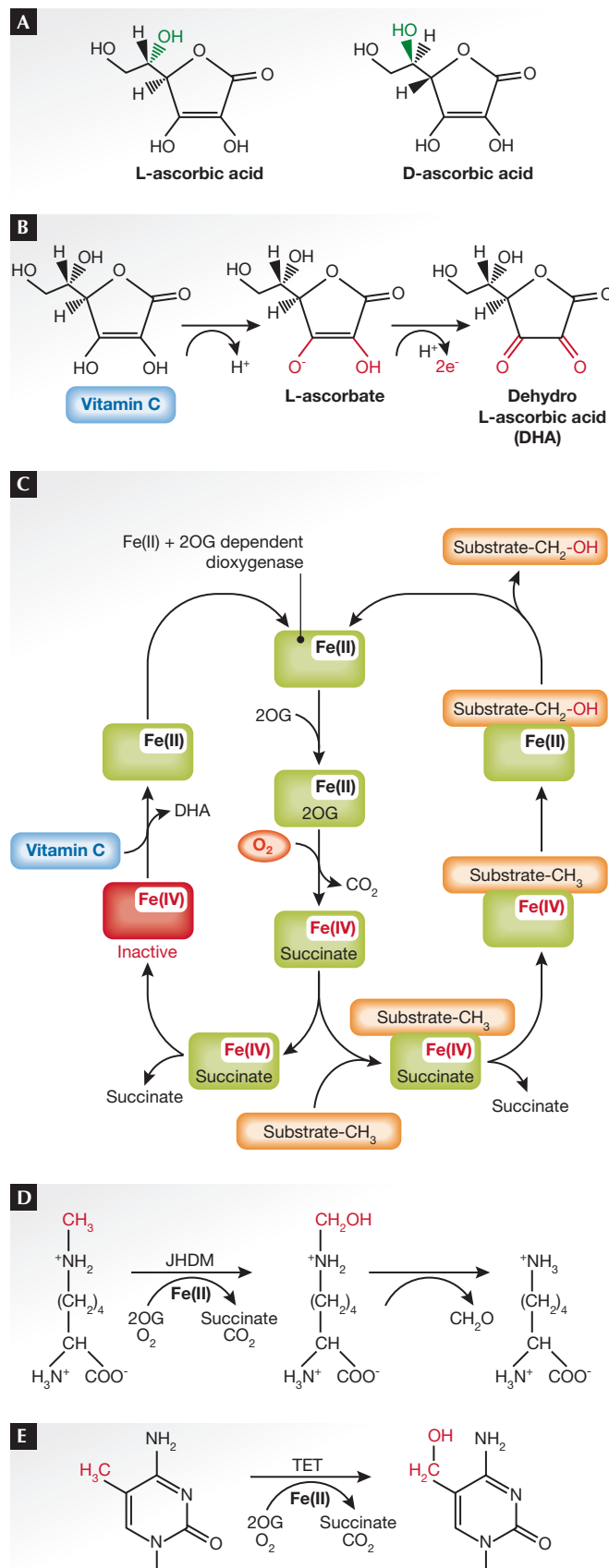
Table 1 | Ascorbate, glutathione and carnitine in common cell culture media and supplements

Reference	Medium/ supplement	L-ascorbic acid	Glutathione	L-carnitine
Sigma Aldrich	DMEM/ Ham's F12	×	×	×
	M2	×	×	×
	M16	×	×	×
	MEM-α	50 mg/l*	×	×
	CMRL-1066	50 mg/l	10 mg/l	×
	Medium 199	0.0566 mg/l*	0.05 mg/l	×
	RPMI 1640	×	1 mg/l	×
Life Technologies	CMRL	50 mg/l	10 mg/l	×
	Advanced DMEM	2.5 mg/l**	1 mg/l	×
	RPMI 1640	×	1 mg/l	×
	GMEM	×	×	×
	Ham's F12	×	×	×
	Neurobasal	×	×	×
	Leibovitz's L-15	×	×	×
	DMEM	×	×	×
	N2 supplement	×	×	×
[7]	KOSR	Yes	N/A	N/A
[98]	B27 (NS21) supplement	×	1 mg/l	2 mg/l

CMRL, Connaught Medical Research Laboratories; DMEM, Dulbecco's modified eagle's medium; KOSR , knock-out serum replacement; MEM, minimum essential medium; RPMI, Roswell Park Memorial Institute; ×, not present; N/A, no data available; *, ascorbic acid sodium salt; **, ascorbic acid phosphate.

with 5mC and paternal chromosomes with 5hmC in preimplantation embryos. Interference with this marking by depletion of *Tet3* in oocytes results in embryonic lethality after implantation that manifests itself with variable penetrance [46]. These findings indicate that Tet enzymes are associated with transitions in cell identity and suggest a role for Tet proteins in the reprogramming of epigenetic patterns. However, none of the Tet genes are essential for development in mice [42,46,48–50], indicating that a potential redundancy or overlapping of functions with other epigenetic pathways might exist.

AlkB family proteins in DNA repair. Computational searches for members of the Fe(II)-2OG-dependent hydroxylase family have revealed homology in the bacterial AlkB DNA repair protein [10]. There are at least nine members of the AlkB protein family in mammals: ABH1–ABH8 and FTO [51,52]. AlkB and its mammalian homologues ABH2 and ABH3 mediate DNA repair by oxidation of 3-methylcytosine and 1-methyladenine [53]. Studies in mice have shown that ABH2 is the crucial enzyme for repairing 3-methylcytosine- and 1-methyladenine-modified DNA [54]. FTO has further been associated with repair of 3-methyl-thymidine in DNA [52]. AlkB enzymes use metal catalysis to oxidize stable methyl adducts that are attached to nitrogens of the heterocyclic bases [53,55,56]. The oxidized methyl groups are released as formaldehyde, and unmodified bases are then regenerated. The mechanism holds similarities to the catalytic mechanism of JHMDs. However, AlkB enzymes have additional domains binding to nucleic acids and distorting DNA structure to flip out bases, making them accessible to chemical reactions [57]. In addition to having an important role in DNA repair, AlkB proteins have also been implicated in repairing RNA in response to exposure to alkylating agents [58]. *In vitro*, ABH2 presents a strong preference for double-stranded DNA contrary to ABH3 that demethylates RNA preferentially [54,58]. Similarly to the JmjC domain protein family, not all AlkB homologues have catalytic activity. Although ABH1 seems to lack measurable DNA/RNA repair activity, it is developmentally regulated and essential for placental development in mice [59]. Mutation of *Abh1* interferes with trophoblast differentiation in mice and results in an increased number of trophoblast stem cells. It has been suggested that *Abh1* modulates histone deacetylation by mutually antagonistic binding with HDAC4 to the essential trophoblast factor Mrj [59] and histone H2A demethylation [60].

FTO is a particularly interesting member of the AlkB family. It has been shown to mediate repair of 3-methyl thymidine in DNA [52]. In addition, mutations in FTO have been correlated with obesity and metabolic disorders in humans. Notably, engineered mutations in FTO have been shown to affect body fat mass in mice [61]. Deletion of FTO leads to postnatal growth retardation [62,63]. Importantly, growth retardation is also observed when FTO is specifically mutated in the brain suggesting a central function in the physiological regulation of energy metabolism and growth. Developmental regulation and phenotypic consequences of mutation of AlkB proteins suggest that some members of this protein family could potentially also have roles in epigenetic regulation, thus, providing functions beyond DNA repair.

Histone demethylases in iPS cell reprogramming

The ability of vitamin C to enhance iPS cell generation has prompted investigations into the mechanism of its action. Notably, the reprogramming process leads to an increased level of reactive oxygen species, suggesting that antioxidant properties of vitamin C might contribute to its effect. However, enhanced reprogramming is not observed with other antioxidants including vitamin B1, reduced gluthathione, sodium selenite, amino-acetylcysteine, resveratrol, α-lipoic acid, vitamin E and L-carnitine [6]. Therefore, the activity of vitamin-C-dependent enzymes is probably more important. Vitamin C promotes cell proliferation and fibroblasts show a striking increase in their lifespan when cultured in its presence. Part of this effect is probably due to counteracting senescence by preventing the activation of the INK4/ARF locus [6], which has been previously described as a road block for iPS cell generation [64]. Inactivating mutations of tumour-suppressor genes or transformation have also enhanced iPS cell formation potentially by increasing cell proliferation [64–66].

To investigate whether vitamin C also facilitates epigenetic changes, Wang *et al* have analysed chromatin modifications during reprogramming in the presence or absence of vitamin C [67]. Their study showed that vitamin C induces a marked reduction in histone H3 Lys 36 dimethylation and trimethylation. Further analysis identified two Fe(II) 2OG-dependent histone demethylases, Jhdm1a and Jhdm1b, as key regulators of Lys 36 demethylation during reprogramming [67]. Combined depletion of Jhdm1a and Jhdm1b by RNA interference impairs reprogramming. Conversely, expression of Jhdm1b and Oct4 is sufficient for reprogramming of fibroblasts to iPS cells [67].

Table 2 | Fe(II) 2-oxoglutarate-dependent dioxygenases involved in epigenetic regulation

Family	Function	Subfamily	Substrate		Enzyme	Reference	Phenotype in development	Reference
JMJC	Histone demethylases		H3K4me1/me2/me3 H3K36me2/me3		No66	[13]	N/A	
		KDM2 JHDM1	H3K36me1/me2		Kdm2a/Jhdm1a FbxL11	[11]	N/A	
					Kdm2b/Jhdm1b FbxL10	[14]	Exencephaly Perinatal death	[99]
		KDM3 JHDM2 JMJD1	H3K9me1/me2		Kdm3a/Jhdm2a Jmjd1a	[12]	Abnormalities in spermatogenesis	[100]
					Kdm3b/Jhdm2b Jmjd1b	[15]	N/A	
					Kdm3c/Jhdm2c Jmjd1c	[16]	N/A	
		KDM4 JHDM3 JMJD2	H3K9me3	H3K36me3	Kdm4a/Jhdm3a Jmjd2a	[22]	N/A	
				N/A	Kdm4b/Jhdm3b Jmjd2b	[23]	N/A	
				H3K36me3	Kdm4c/Jhdm3c Jmjd2c	[24]	N/A	
				H3K9me2	Kdm4d/Jmjd2d	[25]	None	[101]
		KDM5 JARID	H3K4me2/me3		Kdm5a/Jarid1a Rbp2	[26]	Haematopoietic abnormalities (after birth)	[26]
					Kdm5b/Jarid1b	[27]	Embryonic lethal E4.5–E7.5 None	[102,103]
					Kdm5c/Jarid1c Smcx	[28]	Posterior patterning and cardiac defects at E9.5 (male)	MGI
					Kdm5d/Jarid1d Smcy	[28]	N/A	
		KDM6	H3K27 me3/me2		Kdm6a/Utx	[29]	Cardiac defect, growth retardation, cranioschisis and female *in utero* death Haematopoietic defects (after birth) Male: embryonic lethal with variable penetrance Female: embryonic lethal before E11.5	[104,105]
					Kdm6b/Jhdm3d Jmjd3	[30]	Perinatal death	[106]
		PHD	H3K9me2		Phf2	[17]	Perinatal death with variable penetrance Growth retardation	[107]
			H4K20me1 H3K9me1/me2		Phf8	[18] [19]	Cleft lip/palate Mental retardation	[108]
			H4K20me1 H3K9me1/me2 H3K27me2		Kdm7a/Jhdm1d Kiaa1718	[20]	N/A	
		KDM8	H3K36me2		Kdm8/Jmjd5	[21]	Embryonic lethal around E11	[109]
TET	DNA demethylases		5mC 5hmC 5fC		Tet1*	[34]	Slightly smaller body size at birth	[42]
					Tet2*	[34]	Haematopoietic abnormalities (after birth)	[50]
					Tet3	[34]	Perinatal lethality	[46]
ABH	H2A demethylase		Methylated H2A		Alkbh1	[60]	Placental defects	[59]
	DNA demethylases		1meA, 3meC dsDNA		ABH2	[53], [58]	None	[54]
			1meA, 3meC ssDNA/RNA		ABH3	[53], [58]	None	[54]
			3meT ssDNA		FTO	[52]	Reduced size, early death	[63]

*Tet1 and Tet2 double-knockout [43]. ABH, alkylation repair homologue; Alkbh1, alkylation repair homologue 1; ds: double-stranded; E, embryonic day; FTO, fat mass and obesity-associated; JARID, jumonji/AT-rich interaction domain; JHDM, Jumonji family of histone demethylase; JMJC, jumonji C; KDM, lysine demethylase; N/A, no data available; PHD, plant homeodomain finger motif; ss, single-stranded; TET, ten-eleven-translocation.

Notably, reduced levels of histone H3 Lys 36 dimethylation and increased Lys 27 trimethylation at the INK4/ARF locus are observed in cells reprogrammed in the presence of vitamin C, in agreement with reports showing that Jhdm1b regulates cell proliferation and senescence by repressing the INK4/ARF locus through histone H3 Lys 36 demethylation [14,64,68]. The INK4/ARF locus is an important tumour-suppressor locus that encodes three functionally related proteins: p19[ARF], p15[INK4b] and p16[INK4a]. Aberrant mitogenic signals induce expression of the INK4/ARF locus and thereby activate the anti-proliferative retinoblastoma protein and p53 pathways, which mediate cell cycle exit and senescence [69]. INK4/ARF is basally expressed in normal somatic cells and silenced completely in embryonic stem and iPS cells, in which it is also marked by bivalent chromatin [64]. Increased INK4/ARF expression has further been associated with ageing [70]. Somatic cell reprogramming is a proliferation-dependent process and the activity of the INK4/ARF locus has a rate-limiting effect, which is illustrated by the low efficiency of reprogramming of cells from aged tissues. Notably, depletion of mouse *Arf* has been shown to increase the yield of iPS cells, albeit the same effect is not observed during reprogramming of human cells [64].

Reprogramming by using Oct4 and JHDM1B expression in the presence of vitamin C also strongly activates the microRNA (miR) 302/367 cluster [67]. JHDM1B decreases the methylation levels of histone H3 Lys 36 surrounding the Oct4 binding sites of the miR302/367 gene and facilitates gene expression [67]. The miR302/367 cluster regulates pluripotency through inhibition of genes essential for embryonic development and differentiation [71] and is a direct target of Oct4 and Sox2 in embryonic stem cells [72,73]. Consistent with a crucial role in pluripotent cells miR302/367 expression is lost rapidly on differentiation [74]. Notably, expression of the entire miR302/367 cluster is sufficient for reprogramming of fibroblasts, whereby miR367 is essential for activation of Oct4 [75]. This suggests that miR302/367 might be a crucial target of Jhdm1a and Jhdm1b in iPS cell generation.

However, vitamin C has additional functions. When pre-iPS cells, which are trapped at an intermediate state of reprogramming, are treated with vitamin C, conversion to fully reprogrammed iPS cells can be induced [6]. Examination of epigenetic factors that restrict pluripotency has led to the identification of repressive histone H3 Lys 9 methylation marks on transcription factor genes that are required for pluripotent stem cells [76]. Chen *et al* show that, in the presence of vitamin C, histone H3 Lys 9 dimethylation decreases, which correlates with the upregulation of gene expression. This observation suggests that the histone-methylation-mediated epigenetic barrier is sensitive to the presence of vitamin C. Furthermore, inhibition of the Lys 9-specific methyltransferases Suv39h1, Suv39h2, G9a and Setdb1 synergizes with vitamin C in this system. Combined treatment substantially improves the kinetics and efficiency of the reprogramming process. When Setdb1 activity is depleted full reprogramming of pre-iPS cells can also be observed in the absence of vitamin C, suggesting that Setdb1 is crucial for the epigenetic restriction of pre-iPS cells [76]. A targeted RNA-interference-based screen identified the histone demethylase Kdm3b (Jhdm2b) as the primary target of vitamin C. Furthermore, Kdm3a, Kdm4b and Kdm4c also contribute to reprogramming. These results suggest demethylation of histone H3 Lys 9 to be an important step in reprogramming of pre-iPS cells to iPS cells, which is facilitated by enzymes that depend on vitamin C for maintaining

activity. The JmjC-domain-containing H3K27 demethylase Utx has also been identified as a crucial regulator for induction of pluripotency during somatic cell reprogramming [77]. Interestingly, different histone demethylases are required for the reprogramming of fibroblast or pre-iPS cells. Contrary to what is observed in the INK4/ARF locus during the reprogramming of MEFs, upregulation of Jhdm1b (Kdm2b) does not seem to be associated with a reduction in Lys 36 methylation when pre-iPS cells are reprogrammed to iPS cells [76]. Differences in genetic requirements for these reprogramming systems potentially reflect the differentiation state of the starting cell population and the relative time-point in the reprogramming process. Taken together, these results show that regulation of Fe(II) 2OG-dependent histone demethylases is an important factor for reprogramming. Culture conditions without vitamin C apparently limit the activity of these enzymes and thereby interfere with the adjustment of epigenetic patterns.

Tet2 facilitates haematopoietic differentiation

An important insight into the role of Fe(II) 2OG-dependent dioxygenases in cell fate transitions is provided by studies of Tet2 function in haematopoietic differentiation. Mutations of Tet2 are frequently associated with myeloid malignancies. Similarly, disruption of the *Tet2* gene in mice results in an expansion of haematopoietic progenitors and stem cells, and a defect of differentiation of the blood lineages [48–50]. This phenotype is associated with decreased 5hmC suggesting that Tet2-mediated DNA hydroxymethylation is crucial for changing cell identity. Independent support for this view comes from studies of mutations in the isocitrate dehydrogenase genes. *IDH1* and *IDH2* mutations are found in a range of tumour types. These mutations act in a dominant manner and cause a change in substrate specificity that leads to the conversion of 2OG to 2-hydroxyglutarate (2HG; [78,79]). 2HG is not normally present in the cell but acts as an onco-metabolite. It is thought that 2HG inhibits Fe(II) 2OG-dependent dioxygenases by binding in the position that would normally confer 2OG binding [80,81]. An oncogenic mutation in *Idh1* has been associated with impaired haematopoietic differentiation in mice [82]. This phenotype is similar to and can be explained by inhibition of Tet2 function through 2HG [80]. 2HG has also been associated with oncogenic transformation in other tissues. Notably, IDH mutations and 2HG are associated with inhibition of histone demethylation in glioma [83,84]. This observation is consistent with independent work showing that 2HG can inhibit a broad spectrum of α-ketoglutarate-dependent enzymes [85]. Mutations in *IDH1* and *IDH2* might thereby reduce the activity of crucial epigenetic enzymes, thereby inducing errors in cell differentiation that can potentially result in tumour formation [83]. Notably, production of 2HG by oncogenic IDH mutations correlates with epithelial-to-mesenchymal transition in cultured cells [86]. This is an interesting observation that raises the question about the usefulness of 2HG for manipulating the phenotypic state of cells in culture and its potential applications in strategies for directing differentiation (Sidebar A). It is probable that the group of enzymes that are inhibited by 2HG overlap to a large extent with enzymes that are dependent on vitamin C (Table 2; [81]). Although both agents act on various levels, 2HG might enforce a situation that resembles a marked and prolonged deficiency of vitamin C, suggesting mutually antagonistic effects of 2HG and vitamin C on the activity of epigenetic regulators and cellular plasticity.

Vitamin C improves iPS cell quality

Vitamin C has been shown to induce widespread DNA demethylation in human embryonic stem cells. This effect is most clearly observed on genes that are marked by bivalent chromatin modifications and genes that become demethylated during reprogramming [87]. Reprogramming in the absence of vitamin C induces hypermethylation of the imprinted Dlk1–Dio3 locus, which is negatively associated with the quality of mouse iPS cells [7]. The resulting iPS cells are compromised in their ability to form embryonic stem cell-derived mice in tetraploid complementation experiments [88]. Aberrant methylation can be reduced when the relative expression of reprogramming factors is optimized [88]. This suggests that vitamin C could protect from changes induced by suboptimal expression ratios or culture stress. Although vitamin C can prevent methylation defects, it is not sufficient to reverse hypermethylation of the Dlk1–Dio3 locus once established [7].

The Dlk1–Dio3 gene cluster consists of several paternally or maternally imprinted genes. Several pathways contribute to its inactivation in iPS cells. A loss of activating histone H3 acetylation and Lys 4 dimethylation and trimethylation marks, as well as a gain of DNA methylation on the maternal allele, have been observed [89]. DNA hypermethylation has further been shown to require the DNA methyltransferase Dnmt3a. Addition of valproic acid, which is a chemical inhibitor of histone deacetylation can increase histone H3 acetylation and Lys 4 methylation on the silent Dlk1–Dio3 locus but cannot fully rescue the developmental phenotype. By contrast, vitamin C prevents the loss of histone H3 acetylation and Lys 4 methylation resulting in the maintenance of correct Dlk1–Dio3 imprinting [7]. These results emphasize the role of Fe(II) 2OG-dependent dioxygenase function in the regulation of DNA methylation patterns. It is tempting to speculate that Tet1 and Tet2 activities are involved in maintaining DNA methylation patterns in iPS cells. Consistent with this idea, aberrant imprinting marks in the Dlk1 locus have been reported in the progeny of mice carrying mutations in both Tet1 and Tet2 [43]. Recent studies have also provided evidence that Tet1 and Tet2 contribute to reprogramming efficiency [90,91]. However, a complete understanding of the role of Tet enzymes during somatic cell reprogramming requires further studies (Sidebar A).

Concluding remarks and future outlook

Somatic cell reprogramming requires the repression of differentiation genes and the activation of the genes that regulate pluripotency. On the transduction of cells with reprogramming factors, repression of the somatic markers is efficiently achieved but induction of the pluripotency gene network occurs with low efficiency. At this point the epigenetic landscape of the cells is crucial for the success or the failure of the process [92]. Valproic acid is a widely used HDAC2 inhibitor that increases the acetylation state of chromatin and the efficiency of reprogramming [7,75]. This illustrates that compounds promoting changes of the chromatin configuration can potentially enhance reprogramming. Vitamin C contributes to overcoming at least some of the epigenetic constraints towards pluripotency by regulating the INK4/ARF, miR302/367 and Dlk1–Dio3 loci. Furthermore, vitamin C is implicated in a long list of metabolic functions and it could also cause indirect effects through other pathways [4,5,85,93,94].

Recent studies have implicated Fe(II)-dependent oxidative modification activities in normal tissue homeostasis and experimentally induced reprogramming. Histone- and DNA-modifying enzymes

have been identified that contribute crucially to epigenetic regulation in a cell-type- and differentiation-stage-specific manner. Loss of these activities is associated with epigenetic defects and compromised cell differentiation or developmental potential. One of the most important aspects of Fe(II) 2OG-dependent dioxygenases is their susceptibility to environmental factors. These enzymes require vitamin C for maintaining catalytic activity and are inhibited by onco-metabolites [78,79,95]. Insufficient activity of this class of enzymes can lead to irreversible aberrations similar to the defects in DNA methylation patterns observed in some iPS cells. Dietary sources of vitamin C and iron, as well as exposure to onco-metabolites, could have long-term effects on human health. Understanding the molecular links will undoubtedly facilitate future association studies and clarify the wide range of effects attributed to vitamin C (Sidebar A).

In the light of the present literature, the effect of vitamin C in reprogramming might be best explained by a combined enhancement of JHDM and TET activities, which facilitate changing histone methylation and DNA methylation patterns, respectively. Altering epigenetic patterns might be less crucial for traditional cell culture systems that have focused on maintaining cells in a defined state. This could explain the absence of vitamin C from many classical
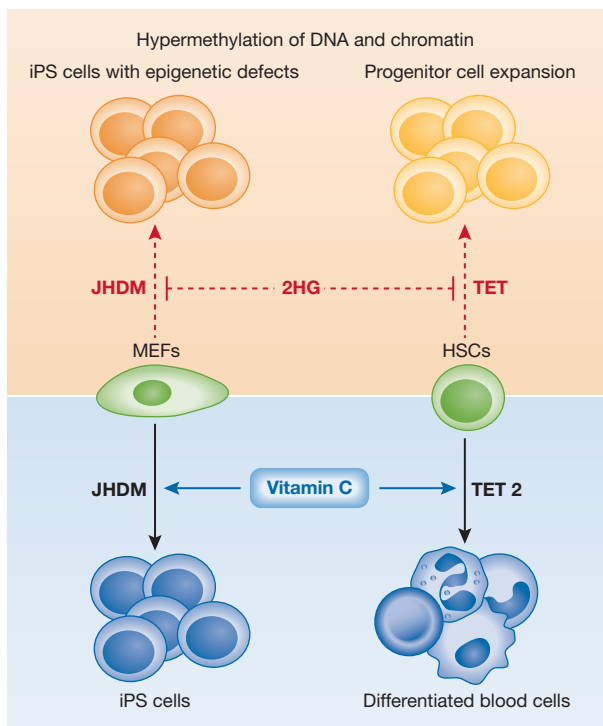
Fig 3 | Summary of ten-eleven-translocation and Jumonji family of histone demethylase function in reprogramming and differentiation. JHDM activity has been shown to be crucial for reprogramming of fibroblasts to iPS cells through heterologous expression of reprogramming factors. Conversely, TET2 has been implicated in facilitating differentiation of haematopoietic stem cells and progenitors. Both activities are dependent on vitamin C and are inhibited by the onco-metabolite 2HG. Loss of these activities, either due to inhibition of 2HG or unphysiologically low levels of vitamin C and iron results in aberrant epigenetic patterns and can block a change in cell identity. 2HG, 2-hydroxyglutarate; iPS, induced pluripotent stem; MEF, mouse embryonic fibroblast; JHDM, Jumonji family of histone demethylase; TET, ten-eleven-translocation.

media formulations. Yet, established cell lines are known to frequently acquire methylation patterns that are not typical of their tissue of origin [96]. Lack of crucial enzyme activities could contribute to a gain of aberrant CpG island methylation. In addition to published literature on embryonic stem cells and MEFs [87,97], it will be interesting to investigate the effects of vitamin C in other culture systems. Similarly, culture under low oxygen pressure could contribute to preserving small amounts of vitamin C in media components such as serum, which would be rapidly oxidized in atmospheric conditions.

Notably, vitamin C and 2HG have opposing effects not only on the activity of Fe(II) 2OG dioxygenases but also on cell fate changes. Whereas vitamin C facilitates reprogramming, 2HG seems to impair differentiation in the haematopoietic system and potentially also in other tissues (Fig 3). Thus, 2HG might be useful as an inhibitor of reprogramming and for stabilizing stem cell fate in culture. Future work will show if 2HG and vitamin C could be potentially useful for regulating stem cell differentiation for applications in regenerative medicine. Furthermore, the development of compounds based on 2OG for potential clinical applications will provide additional tools for the manipulation of cell fates in culture and organisms.

Finally, the puzzling question remains why primates have lost the ability to synthesize vitamin C (Sidebar A). Could environmental influence be selected for when vitamin-C-dependent enzymes perform central functions in cell differentiation? Seasonal changes in vitamin C supply would be predicted to translate into alternating stem cell activity. Low levels of vitamin C might help to stabilize and preserve stem cells in episodes of low metabolic activity similar to stem cell hibernation. If it turns out that evolution has provided for environmental access to stem cell physiology through Fe(II) and 2OG-dependent dioxygenase activity, this could be highly relevant for regenerative medicine. Future work will undoubtedly uncover further molecular details of this important interplay between environment and tissue homeostasis, and provide a clearer picture for potential clinical applications.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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