1 **Chromatin modifications associated with diabetes**

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3
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20 **Abstract**

21 Accelerated rates of vascular complications are associated with diabetes mellitus.
22 Environmental factors including hyperglycemia contribute to the progression of diabetic
23 complications. Epidemiological and experimental animal studies identified poor glycemic
24 control as a major contributor to the development of complications. These studies suggest
25 that early exposure to hyperglycemia can instigate the development of complications that
26 present later in the progression of the disease, despite improved glycemic control. Recent
27 experiments reveal a striking commonality associated with gene-activating hyperglycemic events
28 and chromatin modification. The best characterized to date are associated with the chemical
29 changes of amino-terminal tails of histone H3. Enzymes that write specified histone tail
30 modifications are not well understood in models of hyperglycemia and metabolic memory as well
31 as human diabetes. The best-characterized enzyme is the lysine specific Set7 methyltransferase.
32 The contribution of Set7 to the etiology of diabetic complications may extend to other
33 transcriptional events through methylation of non-histone substrates.

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36

37 **Introduction**

38 Diabetes mellitus is characterised by chronic hyperglycemia induced by loss of insulin
39 producing pancreatic β -cells (Type I diabetes) or progressive loss of insulin sensitivity and β -
40 cell dysfunction (Type II diabetes). It is estimated there are more than 1.7 million sufferers in
41 Australia [1] with worldwide estimates suggesting 150 million patients as of 2003. This is
42 expected to increase to 300 million individuals with the disease by 2025 [2]. Both forms of
43 the disease are associated with accelerated rates of microvascular complications including
44 retinopathy, neuropathy, and nephropathy as well as macrovascular complications such as
45 hypertension, atherosclerosis and stroke. Hyperglycemia presents as a major risk factor for
46 the development of diabetic complications, particularly endothelial dysfunction associated
47 with vascular complications [3]. The vascular damage arising from hyperglycemic insult acts
48 upstream of the overproduction of reactive oxygen species (ROS) by the mitochondrial
49 electron transport chain [4]. Importantly this has been demonstrated to activate the NF κ B
50 pathway leading to inflammatory events in the vasculature [4].

51
52 Several large-scale studies highlight the clinical benefits of strict glycemic control and
53 revealed that early hyperglycemic events instigate the development of diabetic complications
54 that present much later in the progression of the disease [5,6]. In conjunction with studies in
55 experimental animal models and cell culture, these observations imply that a cell may retain a
56 memory of past hyperglycemic events that culminate in altered and persistent gene
57 expression that drives the disease phenotype. This concept has been termed 'metabolic
58 memory' or the 'legacy effect' [5,6]. Current knowledge suggests that chromatin
59 modifications and epigenetic determinants play a key role in the establishment and
60 progression of metabolic memory and persistent gene expression.

61
62 In this review we highlight the epidemiological evidence and experimental studies that
63 demonstrate the detrimental effects of hyperglycemia and support the concept of metabolic
64 memory with a particular focus on Type I diabetes. Furthermore we discuss the current
65 knowledge surrounding epigenetic mechanisms that link hyperglycemia-induced vascular
66 injury to chromatin modifications and altered gene expression as well as metabolic memory.
67 We discuss emerging experimental evidence of changes in genomic methylation and
68 posttranslational modifications such as acetylation and methylation of histone tails, before
69 focusing on perhaps the best characterized methyl-writing enzyme involved in lysine mono-

70 methylation. Additionally we review recent advances in the understanding of Set7-mediated
71 molecular mechanisms that could drive altered gene expression in the diabetic setting.

72

73 **Epidemiological studies**

74 Large-scale studies have highlighted the requirement for strict glycemic control in delaying
75 the onset of diabetic vascular complications. The Diabetes Control and Complications Trial
76 (DCCT) was conducted with a cohort of 1441 Type I diabetic individuals and a mean follow-up
77 of 6.5 years with the aim to compare conventional and intensive glycemic control regimens
78 [7]. Original findings of the DCCT reported that intensive glycemic control reduced the onset
79 and progression of long-term diabetic complications that included neuropathy, nephropathy
80 and retinopathy compared with patients that received conventional therapy [5]. At the
81 conclusion of this trial, a follow-up observational study, the Epidemiology of Diabetes
82 Intervention and Complications (EDIC) examined the long-term effects of the original DCCT
83 cohort. While the DCCT concluded that the development of cardiovascular complications
84 were indistinguishable across intensive and conventionally treated groups, the EDIC trial
85 identified a significantly lower risk of retinal and renal disease in the group that received
86 intensive treatment [8]. At the conclusion of the DCCT, glycosylated hemoglobin (HbA_{1c})
87 levels differed by approximately 2% between the two groups [5]. Intriguingly towards the
88 end of the EDIC study, HbA_{1c} levels of both groups had converged to comparable levels [8].
89 Despite the normalization of HbA_{1c}, the effects of intensive therapy conducted for 6.5 years
90 during the DCCT were persistently beneficial for at least 10 years with regard to
91 microvascular complications. More recently it was reported that patients that received
92 continuous intensive treatment throughout the trials were at significantly lower risk of
93 macrovascular complications including cardiovascular disease and stroke [9], as well as
94 atherosclerosis [10,11]. Long-term benefits of glycemic control have been demonstrated to
95 extend to Type II diabetes mellitus. The UK Prospective Diabetes Study (UKPDS) that
96 compared conventional dietary glucose control with intensive insulin therapy over 10 years
97 initially reported decreased microvascular complications in the intensive therapy group [6].
98 In accordance with the DCCT-EDIC study, 10-year post-trial follow-up revealed lower
99 incidence of macrovascular complications in the intensive therapy group compared to the
100 conventional treatment group [12]. Overall, such findings indicate that previous episodes of
101 poor glycemic control initiate the deleterious effects on the vasculature that persist despite
102 intensive treatment and normalization of blood glucose.

103

104 **Metabolic memory**

105 ***In vivo* evidence from animal models**

106 From observations arising from these clinical trials the term ‘metabolic memory’ was
107 introduced to describe the clinical features of diabetic complications that continue to develop
108 long after exposure to the hyperglycemic insult, despite improved glycemic control [5,8,13].
109 Observations from pioneering studies in animal models also provide strong evidence of this
110 phenomenon. Early studies in dogs with alloxan-induced diabetes demonstrated persistent
111 retinopathy despite a period of 2.5 years of insulin therapy and glycemic control [14].
112 Similarly sucrose-fed diabetic rats that received islet transplantation after 6 weeks of diabetes
113 exhibited reduced progression of retinopathy compared with islet transplantation following
114 12 weeks of diabetes [15]. Another study in rats with streptozotocin-induced diabetes
115 showed that retinal oxidative and nitrative stress resulted from periods of hyperglycemia, and
116 that late reinstitution of strict glycemic control was insufficient to inhibit the progression of
117 diabetic retinopathy [16]. Initiation of good glycemic control after 2 months of hyperglycemic
118 conditions had only partial benefits with regard to oxidative and nitrative stress, whereas a
119 delay in this intervention for 6 months resulted in complete failure to reverse any
120 abnormalities [16]. These observations highlight difficulties in reversing hyperglycemia-
121 induced changes and the need for strict blood glucose control early in the progression of
122 diabetes. A study in mice cells revealed novel insights into the gene transcriptional changes
123 that underlie the phenomenon of metabolic memory with particular regard to inflammation.
124 Aortic endothelial cells from mice subjected to a hyperglycemic clamp for 3h exhibited
125 increased expression of the pro-inflammatory NFκB p65 subunit. This increase in gene
126 expression persisted for 6 days beyond normalization of blood glucose [17]. The p65-NFκB
127 transcription factor drives expression of several pro-inflammatory genes that contribute to
128 the development of diabetic vascular disease [18]. Furthermore p65 expression is increased
129 in diabetic ApoE-null mice aorta and appears to be a central mediator of inflammation in
130 diabetic complications [19].

131

132 **Cell culture experiments demonstrating persistence**

133 Transcriptional changes that govern the pathogenesis of diabetic complications in response to
134 the hyperglycemic insult have begun to be addressed in experiments performed in cell
135 culture. These studies have primarily focused on the vascular endothelium, a key site of
136 hyperglycemia-induced injury [20]. Persistent gene expression was addressed in an early
137 study of cultured primary human endothelial cells exposed to transient high glucose (HG)

138 concentrations. Hyperglycemia induced expression of fibronectin and collagen IV that was
139 maintained despite a return to normoglycemia [21]. Similarly, transient HG increased
140 expression of p65 in primary endothelial cells. Consistent with observations in experimental
141 animal models, the increase in transcription was maintained for up to 6 days following a
142 return to physiological glycemic conditions [17]. Furthermore, expression of downstream
143 p65-NFκB transactivation targets relevant to diabetic vascular injury, monocyte
144 chemoattractant protein 1 (*MCP-1*) and vascular cell adhesion molecule 1 (*VCAM-1*) were
145 significantly increased and remained elevated upon return to normoglycemia [17]. The
146 biochemical basis for the transcriptional response to HG has been delineated by key
147 experiments that implicate the generation of reactive oxygen species (ROS) in the
148 development and persistence of diabetic complications [22]. Increased expression of p65 and
149 downstream transactivation targets was abolished by over-expression of either manganese
150 superoxide dismutase (MnSOD) or uncoupling protein 1 (UCP-1) [17], both of which prevent
151 hyperglycemia-induced superoxide accumulation [4,23]. Similarly the expression of protein
152 markers of hyperglycemia such as protein kinase C-β (PKCβ) and neutrophil cytosol factor 1
153 (p47phox) subunit of NADPH oxidase in endothelial and retinal cells was normalized by
154 inhibition of ROS [24]. Thus the persistence of transcriptional changes following periods of
155 hyperglycemia seems to be imparted by mitochondrial ROS overproduction. Linking these
156 biochemical changes with nuclear mechanisms that govern transcriptional events is a key
157 objective towards understanding glucose-induced transcriptional changes and metabolic
158 memory. Several recent observations have sparked considerable interest in epigenetic
159 mechanisms of gene regulation, particularly histone methylation, which could drive the
160 sustained transcriptional changes observed under transient hyperglycemia.

161

162 **The role of chromatin modifications and epigenetic changes**

163 The term 'epigenetics' was traditionally employed to describe heritable changes in gene
164 expression and cellular phenotype attributable to mechanisms other than alteration of the
165 underlying nucleotide sequence of the DNA [25]. Recently this definition has evolved to
166 include the study of transcriptional regulation with a particular focus on chromatin
167 architecture and the ensuing/preceding interactions between non-genetic factors and
168 chromatin. Chromatin is a dynamic complex of DNA, histone proteins, and numerous
169 modifying complexes, that serves not only to efficiently package DNA, but also to provide a
170 mechanism of transcriptional regulation [25]. Eukaryotic gene transcription is a precisely
171 regulated and multifaceted process, and among other mechanisms is primarily controlled by

172 the degree of chromatin accessibility. Altered states of transcription are initiated or
173 perpetuated by the dynamic structural adaptation of regions of chromatin to confer
174 transcriptionally permissive or repressive configurations [26]. This in turn regulates access
175 and subsequent enzymatic activity of the core transcriptional machinery and associated
176 factors to the underlying DNA sequence. Consequently, the structural state of chromatin
177 determines the transcriptional competency of a gene at a particular locus [27].
178 Environmental factors are proposed to significantly influence the epigenetic signature, and
179 therefore the transcriptional competency of chromatin. Emerging evidence suggests a key
180 role for deregulated epigenetic transcriptional control in the pathogenesis of numerous
181 human diseases including metabolic and inflammatory disorders associated with diabetes.
182 The cellular transcriptional response to various environmental is exemplified by extensive
183 reports of epigenetic transcriptional changes in response to various stimuli and insults in cell
184 culture and experimental models. Thus exposure to environmental factors such as
185 hyperglycemia may lead to the establishment of altered transcriptional states of chromatin
186 that could potentially be retained for the lifetime of the organism. Furthermore there is
187 increasing evidence to indicate that in simple eukaryotes as well as animal models, epigenetic
188 transcriptional states can be meiotically inherited and trans-generationally persistent [28,29].

189

190 **DNA methylation and diabetic complications**

191 A classical example of epigenetic transcriptional control is the covalent post-replicative
192 modification of DNA by the addition of methyl groups to cytosine residues primarily at CpG
193 dinucleotides within the genomic sequence, leading to transcriptional repression. Cytosine
194 methylation within DNA regulatory regions can inhibit transcription by physically precluding
195 the association of DNA and transcription factors [30] and influencing nucleosomal positioning
196 [31,32]. Methylated CpG residues are recognised and bound by several methyl-CpG binding
197 domain proteins which associate with various chromatin modifiers to establish
198 transcriptionally repressed chromatin [33-35]. Limited studies have focused on the role of
199 DNA methylation in the pathogenesis of diabetes, however altered methylation patterns have
200 been reported in patients with diabetes [36].

201

202 Regions of mammalian promoters enriched for CpG dinucleotides (CpG islands) are subject to
203 dynamic methyl modification during development [37]. Studies suggest that *in utero*
204 exposure to a poor nutritional environment might predispose the fetus to the development of
205 diabetes in adult life through aberrant DNA methylation in pancreatic islets of rats [38] and at

206 the *HNF4A* gene locus of CD34+ stem cells from cord blood of neonates [39]. Importantly
207 DNA methylation has been shown to play a role in regulation of insulin expression. Analysis of
208 mouse embryonic stem cells revealed that DNA at the *insulin* (*INS*) promoter was methylated,
209 and became demethylated as the cells differentiated into insulin expressing β -cells.
210 Observation of this specific demethylation extended to the human insulin promoter of
211 pancreatic β -cells [40].

212

213 Several studies have examined the potential role of DNA methylation in the development of
214 diabetic complications, however the significance remains to be completely understood. One
215 study demonstrated global DNA hypermethylation of peripheral blood leukocytes from
216 patients with chronic kidney disease [41]. However analysis of kidney cells exposed to
217 hyperglycemia and renal tissues from Type I diabetic rats showed no difference in DNA
218 methylation of several candidate gene promoters [42]. Conversely numerous candidate genes
219 linked to atherosclerosis were revealed to have altered DNA methylation patterns in
220 endothelial cells [43]. With increased availability of high-throughput technologies for
221 examining the DNA methylome, several observations relevant to diabetic complications have
222 been highlighted by genome-wide studies. Genome-wide analysis of peripheral whole blood
223 DNA methylation patterns from a cohort of 192 Type I diabetics recently identified 19
224 prospective CpG sites associated with the risk of diabetic nephropathy [44]. Global analysis of
225 DNA methylation patterns in peripheral blood monocytes revealed significant changes in
226 patients with increased risk for cardiovascular disease [45]. Furthermore, studies in smooth
227 muscle cells of human atherosclerotic lesions and animal models including ApoE-null mice fed
228 high-fat diets revealed associations between atherosclerosis and global DNA hypomethylation
229 [46-48]. With regard to the development of Type I diabetes, recent genome-wide analysis of
230 DNA methylation of monocytes from monozygotic twins discordant for Type I diabetes
231 identified several diabetes-specific methylation variable positions. Further analysis revealed
232 that the methylation patterns of some of these positions were altered in individuals prior to
233 diagnosis. This suggests that they arise early in the pathology of Type I diabetes and unlike
234 aforementioned examples cannot be explained by post-disease cellular dysfunction [49].

235

236 **Chemical diversity of histone tail modifications**

237 Traditionally considered as primarily passive structural elements histone proteins are now
238 recognised as integral components of chromatin fundamental to the regulation of gene
239 expression [50,51]. Underlining their importance to nuclear regulation, histones are among

240 the most highly conserved proteins through the eukaryotic domain [52]. Each histone
241 peptide possess an unstructured N-terminal tail composed of 15-44 amino acids that protrude
242 from the nucleosome to provide exposed surfaces for interaction with other proteins. The tail
243 domains harbor multiple lysine and arginine residues that are substrates for a variety of
244 covalent post-translational modifications including methylation, acetylation, phosphorylation
245 and ubiquitination [53]. Many of these modifications are associated with distinct
246 transcriptional states and can generate synergistic or antagonistic effects in conjunction with
247 other histone modifications to control transitions between transcriptionally active and
248 inactive chromatin [27].

249
250 The wealth of post-translational modifications to the histone tail suggests a complicated and
251 combinatorial epigenetic language that co-ordinates structural and therefore transcriptional
252 changes. Indeed several observations indicate that acetylation of particular histone tail
253 residues can complement the effects of other histone modifications such as methylation on
254 gene transcription [54]. Enzymatic transfer of an acetyl group to the ϵ -amino group of histone
255 H3 and H4 lysine tails by histone acetyltransferases (HATs) has been correlated with
256 transcriptional activation [55] most likely through alterations of electrostatic interactions
257 [56]. By contrast, removal of the acetyl group by histone deacetylases (HDACs) is associated
258 with transcriptional repression. Accordingly euchromatin is highly enriched for acetylated
259 histones, whereas hypoacetylated histones are observed predominately at transcriptionally
260 inactive heterochromatin [57,58]. Such broad observations have been proposed to reflect
261 transcriptional competency rather than active transcription of such regions [59].
262 Nonetheless, hyperacetylation of certain histone lysine residues, particularly K9 and K14 of
263 histone H3, has been thoroughly demonstrated as characteristic of promoter and enhancer
264 regions of genes in active transcriptional states and several enzymes and complexes
265 responsible for the addition and removal of the acetylation mark have been described [60-
266 63]. Recent studies have explored the relationship between histone hyperacetylation and
267 gene expression under diabetic conditions. Cultured primary human endothelial cells
268 exposed to hyperglycemic conditions displayed increased expression of p300
269 acetyltransferase and enrichment of this enzyme to the promoters of HG-responsive genes
270 such as fibronectin [64]. Hyperacetylation of histone H4 at the promoter enhances expression
271 of the *INS* gene [65]. Furthermore the β -cell specific transcription factor PDX1 was shown to
272 interact with p300 to mediate proinsulin gene expression [66]. Thus histone acetylation is
273 involved in both regulation of insulin secretion and damage associated with hyperglycemia.

274 Therapeutic targeting of the enzymes that regulate these processes may therefore alleviate
275 some aspects of the disease. To this end the anti-inflammatory effects of HDAC inhibition
276 have recently been investigated. Genome-wide mRNA sequencing of human aortic endothelial
277 cells revealed decreased expression of numerous inflammatory cytokines and chemokines
278 such as *IL-6*, *IL-8*, *MCP-1* and *MIF* and cell adhesion molecule *ICAM-1* upon treatment with the
279 HDAC inhibitor Trichostatin A (Balcerczyk *et al.*, manuscript in preparation). The aim of this
280 review is not to provide an exhaustive description of each histone modification, which have
281 recently been reviewed [67], but instead we focus on key relevant histone modifications in
282 context to hyperglycemia, metabolic memory and its persistence. Perhaps the best
283 characterized enzyme relevant to diabetic complications is the Set7 methyltransferase in
284 primary culture experiments and small animal models of hyperglycemic memory.

285

286 **Histone methylation**

287 Histone methylation modifications catalysed by histone methyltransferases (HMTase) define
288 and direct the formation of distinct chromatin regions [68,69]. Contrasting histone
289 acetylation, methylation of lysine and arginine residues can be associated with both gene
290 activation and repression, depending on the residue modified [69]. Most HMTase enzymes
291 that specifically methylate lysine residues are classified as members of the SET family of
292 proteins. Responsible for the methyltransferase activity is the presence of the evolutionarily
293 conserved, 130-amino acid SET domain initially identified in three proteins shown to be
294 required for maintenance of the expression of genes important to normal development in
295 *Drosophila melanogaster*; positive effect variegation suppressor **S**U(VAR)3-9, the polycomb
296 group protein **E**nhancer of zeste and the homeobox gene regulator **T**rithorax [70-73].
297 Identification of this common motif has rapidly increased the number of genetically and
298 biochemically identified modifiers of histone proteins across several organisms. Multiple
299 SET-domain HMTases have been characterized that methylate histone lysine residues in
300 mammalian cells (Table 1). SET domain HMTases contribute to the regulation of
301 transcriptional activity by methylating histone proteins within chromatin to maintain active
302 and repressed transcriptional states through various nuclear processes. These modifications
303 occur on several lysine residues within the N-terminal tail of histones H3 and H4, and the
304 effect on transcription is dependent on both the specific lysine residue modified and the
305 number of methyl groups covalently assigned. The enzymes responsible generally exhibit
306 strong substrate and product specificity. Methylation of lysine residues 4, 36 and 79 of
307 histone H3 are associated with active gene expression [25,74,75], while methylated lysine

residues 9 and 27 of histone H3 and lysine 20 of histone H4 are generally associated with transcriptionally silenced or inactive chromatin [25,75,76]. Recent genetic examination of genes encoding histone lysine methyltransferases in patients with Type 1 diabetes suggests the importance of an inherited genetic component for the risk of diabetic complications. In the well-characterised Finnish Diabetic Nephropathy Study (FinnDiane) cohort, a polymorphism in the *SUV39H2* gene that encodes a H3K9-specific methyltransferase was found to be associated with diabetic retinopathy [77]. Though this gene has not previously been linked to diabetic complications, it is a close homologue of *SUV39H1* that has been implicated in hyperglycemia-induced inflammation [17].

Table 1. Mammalian Histone Lysine Methyltransferase Enzymes

Histone Target	Lysine methyltransferase		
	Monomethylation	Dimethylation	Trimethylation
H3K4	Set7	Smyd3, SETMAR, NSD3	Smyd3, MLL, SETD1A, SETD1B
H3K9	G9a, Eu-HMTase1	G9a, Eu-HMTase1	SETDB1, SETDB2, SUV39h1, SUV39h2
H3K27	EZH2, EZH1, G9a	EZH2, EZH1, G9a, NSD3	EZH2, NSD3
H3K36	NSD1, ASH1L	NSD1, Smyd2, SETMAR, ASH1L	SETD2, ASH1L, NSD2
H3K79	DOT1L	DOT1L	DOT1L
H4K20	Pr-Set7(Set8), NSD2	Pr-Set7(Set8), NSD1, SUV4-20	NSD2, SUV4-20

332 **Set7 methyltransferase**

333 A SET-domain HMTase has recently received considerable attention for its ability to catalyse
334 the activating H3K4 monomethylation (H3K4me1) reaction. Sharing little sequence
335 homology with any of the six previously described yeast SET-domain containing proteins, the
336 enzyme was termed Set7 [78] (also described as Set9) [79]. Initial characterization
337 demonstrated the ability of Set7 to stimulate GAL4-VP16-activated luciferase expression in
338 cancer cell lines, implying a role in activator-induced transcription [79]. To this end, several
339 functions attributable to Set7 activity have been observed *in vitro* which may be important to
340 understanding the mechanism behind the transition between transcriptionally inactive and
341 active chromatin. Set7-mediated H3K4 methylation inhibits Suv39h1-mediated H3K9
342 methylation and facilitates acetylation of both H3 and H4 by the acetyltransferase p300 [80].
343 Furthermore, association of the NuRD chromatin remodeling and deacetylase complex with
344 the histone H3 tail is inhibited by H3K4 methylation, but not H3K9 methylation [79]. This
345 antagonistic paradigm suggests that Set7-mediated H3K4me1 has the ability to maintain an
346 active chromatin state by preventing both NuRD- and HP1-mediated (H3K9 methylation-
347 dependent) transcriptional silencing. Interestingly, methylation of H3K4 by Set7 did not have
348 the same effect on H3K9 methylation by G9a [79]. This observation excludes Set7-mediated
349 H3K4me1 as a global regulator of H3K9 methylation and by extension global transcriptional
350 repression.

351

352

353 **Set7 methyltransferase regulates metabolic memory and inflammatory diabetic** 354 **complications**

355 Several studies associate Set7 methyltransferase activity at H3K4 with gene activation under
356 diabetic conditions in pancreatic cells [81-83], monocytes [84] and endothelial cells [17,85].
357 Recently HG concentrations were demonstrated to promote nuclear localization of Set7 in
358 human endothelial cells [86]. Reduced H3K4me1 enrichment at discrete hyperglycemia-
359 responsive loci following Set7 depletion has been reported in models of hyperglycemic
360 variability. Following transient exposure of endothelial cells to HG, persistent transcriptional
361 activation of p65 (encoded by the *RELA* gene) and several inflammatory NFκB-dependent
362 genes paralleling enrichment of Set7 and H3K4me1 at the *RELA* promoter was observed upon
363 subsequent return to normoglycemia. This response was sustained for 6 days implying a
364 memory of recent exposure to the metabolic insult. Set7 knockdown abrogated *RELA*
365 promoter H3K4me1 enrichment and p65 mRNA and protein expression in response to

transient and prior HG in both human and bovine endothelial cells. Accordingly the transcriptional persistence of p65 and its downstream transcriptional targets was attenuated [17,85]. Furthermore, sustained reduction of H3K9 methylation on the *RELA* promoter following transient and prior hyperglycemia was observed [85]. Methylation H3K9 and H3K4 have previously been demonstrated to inhibit each other [78] and the occlusion of H3K9 methylation by Set7-mediated methylation of H3K4 may represent another mechanism by which Set7 maintains an active chromatin state at the *RELA* promoter [85].

Experimental evidence suggests that Set7 may function as a co-activator of transcription through direct interaction with another transcriptional activator and specific co-recruitment to promoters. Monocyte transcription of a subset of TNF- α -inducible NF κ B-dependent inflammatory genes was compromised by depletion of endogenous Set7 through a different mechanism than previously reported for glucose-stimulated endothelial cells. Expression of TNF- α -inducible p65-dependent *MCP-1*, *IL-8* and *TNF- α* was shown to be dependent on Set7 expression [84]. TNF- α -stimulated cells displayed increased *MCP-1* expression concomitant with enrichment of Set7, p65 and H3K4 methylation at the *MCP-1* and *TNF- α* promoters. Targeted Set7 disruption using shRNA impaired this response, namely the promoter-specific recruitment of p65. Microarray analysis of TNF- α -stimulated Set7 knockdown monocytes revealed altered transcriptional induction of over 25% of TNF- α -regulated genes. Interestingly, the level of p65 expression detected by western blot was similar across control and Set7 deficient cells [84]. This observation indicates that the effects on downstream transcription targets were not a result of attenuated *RELA* transcriptional activation, as was the case in the response of endothelial cells to glucose stimulation. Rather Set7 appears to act as a co-activator of a subset of p65-mediated transcription of TNF- α -sensitive genes. While these seemingly discordant findings may reflect differences across cell types, it is likely that Set7 has a dual function in establishment of the pro-inflammatory phenotype.

Intriguingly the p65 subunit was recently identified as a novel substrate for Set7-mediated methylation. Studies have demonstrated this modification to have implications for p65 transactivity and downstream transcription of NF κ B-dependent genes. This is a significant finding given the enzymes's reported role in persistent transcriptional activation of the *RELA* promoter in endothelial cells, and further implicates Set7 in regulation of NF κ B-dependent inflammatory pathways. A recent report describes complete abolition of *in vitro* p65 methylation by a single point mutation at K37, indicating this residue to be the lysine targeted

400 for modification [87]. Methylation dependent regulation of p65 may represent an integral
401 control point with regard to the expression of a variety of genes.

402

403 While several known examples of posttranslational p65 modification exist, the biological
404 response to Set7-mediated methylation of p65 remains to be fully established. Relative to the
405 unmodified protein, p65 methylated at K37 is restricted to the nucleus and enriched at a
406 subset of NF κ B-dependent promoters in response to TNF- α stimulation [87]. Conversely,
407 examples of this modification have also linked Set7 to transcriptional repression. Two
408 alternative sites at K315 and K316 that are subject to Set7-dependent methylation have been
409 reported, modification of which can result in negative regulation of p65 transactivity *in vivo*
410 [88]. Subsequent kinetic analysis reveals that the K37 methylation event occurs prior to K315
411 and K316 methylation, and may be the preferential methylation site [87]. Nevertheless p65
412 appears to contain Set7-dependent regulatory sites for both transcriptional activation and
413 repression of downstream gene targets. Overall Set7 appears to be a key mediator of the pro-
414 inflammatory response to hyperglycemia through both increased p65 expression via
415 promoter H3K4 methylation enrichment [17] and post-translational methylation of distinct
416 lysine residues within the p65 subunit of NF κ B [87,88]. In support of Set7-dependent
417 regulation of p65 transactivity, recent massive parallel sequencing analysis of a model of Set7
418 depletion in human endothelial cells implicates deregulated p65 transactivity in the
419 differential expression of numerous genes, some of which have associations with
420 inflammation and diabetic complications (unpublished data) (Figure 1).

421

422 A similar co-activational role has been described in pancreatic β -cells with regard to Set7
423 recruitment and enrichment of H3K4 methylation at the *INS* promoter. Co-
424 immunoprecipitation and GST pull-down assays in conjunction with ChIP assays demonstrate
425 the recruitment of Set7 and subsequent methyltransferase activity to be mediated through
426 direct interaction with transcriptional activator; pancreatic duodenal homeobox-1 (PDX-1).
427 Depletion of either Set7 or PDX-1 expression significantly decreased transcription of a
428 reporter construct driven by an intact *INS* promoter [83]. Significant reduction in
429 transcription of a subset of glucose responsive genes was observed in mouse β -cells and
430 primary islets *in vitro*, following siRNA-mediated Set7 depletion [81]. Unexpectedly, this
431 transcriptional diminution correlated with reduced H3K4me2 enrichment at the proximal
432 promoter regions of *Ins1*, *Ins2* and *Slc2a2*, while no change to the H3K4me1 status was
433 reported. The authors noted that these results do not necessarily rule out Set7's function as a

434 monomethylase and raise the possibility of another closely linked HMTase capable of
435 completing the di-methylation reaction on the enzymatic product of Set7 [81]. Importantly
436 the overall experimental observations highlight the necessity of Set7 function in β -cells for
437 proper insulin production and secretion.

438

439 A recent study implicates Set7 as a co-activator of TGF- β -induced expression of extracellular
440 matrix genes relevant to the development of diabetic nephropathy [89]. Under hyperglycemic
441 conditions, increased expression of fibrotic genes *Col1a1*, *CTGF* and *PAI-1* in rat mesangial
442 cells was demonstrated to correlate with enrichment of promoter H3K4me1 and Set7
443 recruitment. Increased gene expression and Set7 recruitment were attenuated by both pre-
444 treatment with a TGF- β -specific antibody and targeted Set7 disruption by siRNA transfection
445 [89]. *Col1a1* [90], *CTGF* [91] and *PAI-1* [92] are *bona fide* transactivation targets of SMAD3, a
446 key mediator of renal fibrosis and inflammation [91,93,94]. Indeed deletion of SMAD3
447 conferred protection against fibrogenesis in a rodent model of diabetic nephropathy [95].
448 Under diabetic conditions, critical mediators of diabetic complications AGEs [91,96] and
449 Angiotensin II [93,97] can also activate SMAD signaling pathways to stimulate extracellular
450 matrix production via TGF- β -dependent and independent mechanisms. Thus it is tempting to
451 speculate a mediatory role for SMAD3 in the recruitment and specific enrichment of Set7 to
452 activating genes targets in response to TGF- β stimulation to potentiate the H3K4me1 reaction.
453 Interaction between Set7 and SMAD3 has not been demonstrated experimentally, but may
454 provide another example of transcription factor-Set7 co-activation possibly via a methylation-
455 dependent mechanism. Disruption of a potential Set7/SMAD3 interaction may provide a
456 novel therapeutic target to alleviate renal complications associated with the diabetic milieu.
457 Taken together, these findings provide evidence of co-recruitment of Set7 to specific genes
458 and suggest that Set7 may function in complex with transcriptional co-activators. For instance
459 ordered recruitment of Set7 and other transcriptional regulatory proteins indicate that Set7
460 co-assembles as a component of a pre-initiation complex required for transcriptional
461 activation of *collagenase I* [98].

462

463 **Set7 methylates non-histone substrates**

464 As highlighted by reports of Set7-dependent post-translational methylation of p65 lysine
465 residues, the molecular events mediated by Set7 in response to hyperglycemia might not be
466 restricted to histone methylation. Analysis of Set7 methyltransferase activity toward lysine
467 residues of non-histone proteins has revealed previously unknown mechanisms by which

468 Set7 may contribute to transcriptional regulation and other cellular processes [99].
469 Numerous additional substrates have recently been described sparking considerable interest
470 in the characterization of these methylation events and the identification of novel lysine
471 targets [100]. Adding to the complexity of the role of Set7 in transcriptional regulation,
472 transcription factors predominate the list of confirmed Set7 substrates. The *in vivo*
473 consequences of post-translational methylation on most of these targets remain to be fully
474 understood. However numerous observations of Set7 methyltransferase activity-dependent
475 transcription factor stability and activity have been reported, and several examples are
476 described in Figure 2.

477

478 Recent studies suggest novel mechanisms of Set7 recruitment to individual promoters by
479 Set7-transcription factor binding and modification, providing further evidence of a co-
480 activational role. Several recently identified Set7 substrates display modified biological
481 activity following Set7-mediated methylation including p53 [101], DNMT1 [102] and ER- α
482 [103]. This post-translational methylation can result in altered expression of downstream
483 transcriptional targets. For instance the TFIID transcription factor complex component
484 TAF10 is methylated at K189 by Set7 [104]. While this modification does not affect the
485 incorporation of TAF10 into TFIID, methylated TAF10 displays increased affinity for RNAPII
486 suggesting a role for this modification in regulating preinitiation complex formation. To this
487 end, transcription of several known TAF10-dependent genes was enhanced by methylation of
488 TAF10 [104]. While this report highlights an example mechanism whereby Set7 is brought
489 within close proximity of an activating promoter, subsequent effects on H3K4 methylation
490 remain unresolved.

491

492 Sequential Set7 recruitment to histone H3 following TAF10 promoter binding remains to be
493 demonstrated. Recent reports suggest this indeed to be the case with an interaction between
494 Set7 and another recently characterized substrate. STAT3 is methylated at K140 by Set7, and
495 de-methylated by LSD1 [105]. This modification both positively and negatively regulates the
496 expression of a multitude of STAT3-dependent genes in response to IL-6 stimulation, and can
497 be disrupted by Set7 depletion or cells expressing STAT3 R140 mutant proteins.
498 Furthermore, STAT3 promoter occupancy of a subset of these genes was shown to be
499 dependent on Set7 methyltransferase activity. This regulation is promoter specific as not all
500 STAT3-dependent IL-6 responsive promoters exhibited methylated STAT3 enrichment [105].
501 IL-6 is considered to play a central role in the development of diabetic complications

502 including diabetic nephropathy [106,107]. Circulating IL-6 levels are reportedly elevated in
503 patients with type 2 diabetes [108], are associated with poor glycemic control [109]. Recent
504 microarray analysis of diabetic kidneys revealed significant up-regulation of genes in the
505 JAK/STAT pathway within glomeruli and tubules [110]. Furthermore components of the
506 diabetic milieu including ROS [111], angiotensin II signaling [105] and AGEs [112] have been
507 experimentally demonstrated to activate this pathway in kidney cells. In a study of diabetic
508 mice with compromised STAT3 activity, extracellular matrix-associated TGF- β and type IV
509 collagen expression as well as mesangial cell proliferation were inhibited. Attenuation of the
510 diseased phenotype extended to a reduction in the expression of pro-inflammatory factors IL-
511 6, ICAM-1, MCP-1 and nuclear translocation of NF κ B [113]. Given the responsivity of Set7 to
512 hyperglycemia and ROS and its transactivity-modifying capacity of STAT3, it is reasonable to
513 speculate a role for the methyltransferase in the development and progression of diabetic
514 nephropathy through this pathway.

515

516 The *SOCS3* gene was strongly up-regulated following Set7 knockdown, and displays increased
517 STAT3 enrichment at the promoter [105]. Furthermore it was demonstrated that Set7 is
518 recruited to the *SOCS3* promoter after STAT3 recruitment. This sequence of events may
519 function to clear STAT3 from the activating promoter and simultaneously potentiate
520 methylation of H3K4 [105]. Set7/STAT3-regulated expression of *SOCS3* may hold therapeutic
521 relevance for diabetic nephropathy as *SOCS3* is a negative-feedback regulator of the
522 JAK/STAT pathway [114], specifically STAT3 activation [115]. To this end, over-expression of
523 *SOCS3* inhibited the hyperglycemia-induced activation of STAT-responsive inflammatory
524 genes *MCP-1*, *ICAM-1* and *IL-6* [116].

525

526 A similar interaction between Set7 and the androgen receptor (AR) has been described [117]
527 as a methylation event important for AR activation and recruitment to androgen-regulated
528 promoters. This interaction may facilitate H3K4me1, as depletion of endogenous Set7 in
529 cancer cells reduced H3K4me1 enrichment at proximal and enhancer regions of the
530 androgen-dependent *PSA* promoter [117]. This observation holds relevance for diabetic
531 complications, as hyperandrogenemia is associated with β -cell dysfunction [118,119]. In
532 support, a recent study in a mouse model reported increased systemic oxidative stress
533 stemming from testosterone treatment was AR-dependent. Furthermore female mouse
534 pancreatic islets treated with streptozotocin displayed increased β -cell destruction [120].

535 Further investigation may reveal Set7 as a potential therapeutic target in the treatment of
536 hyperandrogenemia and the accompanying insulin resistance.

537

538 **Conclusion**

539 Estimates indicate that the incidence of diabetes mellitus and associated complications is
540 rapidly increasing. It is therefore imperative that strategies to combat progression of
541 detrimental complications are established. Knowledge of the molecular etiology of diabetes
542 mellitus is paramount to understanding the link between environmental factors such as
543 nutrition and altered/persistent gene expression that drive the disease phenotype. Clinical
544 and experimental studies have revealed the deleterious consequences of exposure to
545 hyperglycemia, and clearly highlight the importance of strict glycemic control. The concept
546 that a cell retains a metabolic memory of prior exposure to hyperglycemic events was a
547 seminal hypothesis that arose from epidemiological studies of diabetic patients. This concept
548 is further exemplified by experimental studies that demonstrate hyperglycemia-induced
549 changes in gene expression that persist despite a return to physiological glucose
550 concentrations.

551

552 The dynamic processes that govern epigenetic regulation of chromatin confer an additional
553 layer of transcriptional control that links gene expression with environmental signalling.
554 Experimental evidence demonstrates that hyperglycemia can induce epigenetic modifications
555 to chromatin structure via various nuclear processes. Specific histone-modifying enzymes
556 have been implicated, and have been shown to regulate the expression of inflammatory genes
557 in cells of vascular origin. In these models of glycemic memory, the Set7 methyltransferase
558 was shown to engage the histone H3 tail to establish H3K4me1 at the pro-inflammatory *RELA*
559 promoter, a modification that persists despite normalisation of glucose conditions. Certainly
560 other histone modifications and the responsible enzymes are expected to be involved in
561 transcriptional changes induced by elevated glucose concentrations, and are likely to be
562 explored in models of glycemic memory. Characterisation of these events will be greatly
563 assisted by recent advances in powerful genome-wide technologies such as massive parallel
564 sequencing and bioinformatics tools. For instance genome-wide analysis of primary vascular
565 cells exposed to HG concentrations has revealed relationships between acetylated histone
566 tails, DNA methylation and expression of genes associated with endothelial dysfunction [121].

567

568 The role of Set7 in gene activational events under diabetic conditions extends to pancreatic
569 and monocytic transcriptional regulation that also hold relevance for diabetic complications.
570 Additionally, recent and rapid expansion of substrates alternative to the histone H3 tail may
571 provide further evidence of the enzyme's involvement in the pathogenesis of diabetic
572 complications. Defining non-histone Set7 substrates is critical to understanding the role of Set7 in
573 transcriptional regulation in health and disease. This avenue of investigation may uncover novel
574 mechanisms by which Set7 is recruited to activating promoters at specific loci to execute the H3K4
575 methylation reaction. By the same token, Set7-regulated protein activity and stability, and
576 consequential effects on transactivation potential is another mechanism of transcriptional control
577 that may be applicable to diabetic complications. To attenuate the burden of diabetic
578 complications it may soon be possible to interrupt the epigenetic pathways that promote
579 diabetic complications such as vascular injury. Specific targeting of the enzymes implicated in
580 chromatin structure and gene function such as Set7 provides potential for future epigenetic
581 therapy for diabetic complications. However several challenges to this approach are apparent
582 in the multiplicity of modifications that govern gene expression. The combinatorial nature of
583 histone modification patterns at individual loci and genome-wide will be rapidly unveiled
584 with advances in sequencing technology. It is anticipated that this will strongly build upon
585 key findings from *in vitro* studies of glucose- and ROS-induced vascular injury and potentially
586 lead to therapeutic inhibition of methyl-writing and methyl-erasing enzymes to attenuate
587 complications of diabetes.

588

589 Another strategy to abrogate the effects of hyperglycemic exposure is inhibition of ROS
590 overproduction. Oxidative stress plays a pivotal role in the development of complications of
591 diabetes by activating numerous pathogenic pathways [122]. Furthermore increased ROS
592 production inactivates 2 athero-protective enzymes eNOS [123,124] and prostacyclin
593 synthase [125]. The significant reduction of eNOS and prostacyclin synthase activity
594 attributable to hyperglycemia-induced ROS overproduction was attenuated by treatment with
595 a superoxide dismutase/catalase mimetic [23]. Similar strategies of ROS inhibition have been
596 demonstrated to prevent the development of complications including cardiomyopathy
597 [126,127], nephropathy [128,129], retinopathy [130] and neuropathy [131] in diabetic mice.

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Figure 1 Massive parallel sequencing analysis of a model of Set7 depletion in human endothelial cells. Deregulated p65 transactivity is implicated in differential expression of numerous genes, some of which have associations with inflammation and diabetic complications. Vertical lines categorise the cellular location of protein products of the deregulated genes in the network: extracellular, membrane, cytoplasm, nucleus and unspecified. The single arrow going toward p65 marks the interaction with the Set7 methyltransferase. Red circles in the right corner of a gene indicate increased expression and blue circles indicate decreased expression. This transcription factor centric network was generated using the commercial software MetaCore™ version 6.8 build 30387 (GeneGo, Inc) which draws from the manually curated MetaCore database.

Figure 2 Examples of transcription-associated protein substrates for the methyltransferase reaction catalysed by Set7. Several of these modifications may contribute to a positive transcriptional outcome. Set7-mediated methylation of K142 destabilizes DNMT1 protein by facilitating polyubiquitination and subsequent proteasome-mediated degradation [102]. By contrast methylation of p53 at K372 and subsequent acetylation by Tip60 acetyltransferase results in increased stability of the p53 protein, thereby influencing p53-mediated gene expression [132]. Methylation of p65 at K37 methylated p65 is restricted to the nucleus and is enriched at a subset of NFκB-dependent promoters in response to TNF-α stimulation. Methylation at K140 and K630 of STAT3 and AR respectively results in co-recruitment of Set7 to target gene promoters as a co-activator of transcription. STAT3 is reportedly di-methylated by Set7 [105].

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704

705 **Declarations**

706 None
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708 References

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