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REVIEW

Islet biology, the *CDKN2A/B* locus and type 2 diabetes risk

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Abstract Type 2 diabetes, fuelled by the obesity epidemic, is an escalating worldwide cause of personal hardship and public cost. Diabetes incidence increases with age, and many studies link the classic senescence and ageing protein $p16^{INK4A}$ to diabetes pathophysiology via pancreatic islet biology. Genome-wide association studies (GWASs) have unequivocally linked the *CDKN2A/B* locus, which encodes p16 inhibitor of cyclin-dependent kinase ($p16^{INK4A}$) and three other gene products, p14 alternate reading frame ($p14^{ARF}$), $p15^{INK4B}$ and antisense non-coding RNA in the *INK4* locus (ANRIL), with human diabetes risk. However, the mechanism by which the *CDKN2A/B* locus influences diabetes risk remains uncertain. Here, we weigh the evidence that *CDKN2A/B* polymorphisms impact metabolic health via islet biology vs effects in other tissues. Structured in a bedside-to-bench-to-bedside approach, we begin with a summary of the evidence that the *CDKN2A/B* locus impacts diabetes risk and a brief review of the basic biology of *CDKN2A/B* gene products. The main emphasis of this work is an in-depth look at the nuanced roles that *CDKN2A/B* gene products and related proteins play in the regulation of beta cell mass, proliferation and insulin secretory function, as well as roles in other metabolic tissues. We finish with a synthesis of basic biology and clinical observations, incorporating human physiology data. We conclude that it is likely that the *CDKN2A/B* locus influences diabetes risk through both islet and non-islet mechanisms.

Keywords Ageing · *ANRIL* · Beta cell mass · *Cdkn2A* · *Cdkn2B* · *CDKN2B-AS* · Insulin secretion · Oncogene · p14 · $p14^{ARF}$ · p15 · $p15^{INK4B}$ · p16 · $p16^{INK4A}$ · Pancreatic beta cell · Proliferation · Review · Senescence · Tumour suppressor

Abbreviations

ANRIL	Antisense non-coding RNA in the *INK4* locus
ARF	Alternate reading frame
BMI1	B cell-specific Moloney murine leukaemia virus insertion site 1
CBX7	Chromobox homologue 7
CDK	Cyclin-dependent kinase
CDKN2	Cyclin-dependent kinase inhibitor 2
DPP	Diabetes Prevention Program
E2F	Early region 2 transcription factor
eQTL	Expression quantitative trait locus
EZH2	Enhancer of zeste 2
GWAS	Genome-wide association study
HNF	Hepatic nuclear factor
INK	Inhibitor of cyclin-dependent kinase
lncRNA	Long non-coding RNA
MDM2	Mouse double minute 2 homologue
MLL1	Mixed lineage leukaemia protein 1
P38MAPK	p38 mitogen-activated protein kinase
PTEN	Phosphatase and tensin homologue
Rb	Retinoblastoma
SNP	Single nucleotide polymorphism
WIP1	Wild-type p53-induced phosphatase 1

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Summary points

- Non-coding polymorphisms near the *CDKN2A/B* locus influence risk of several type 2 diabetes-related forms of diabetes
- The *CDKN2A/B* locus encodes $p16^{INK4A}$, $p15^{INK4B}$, $p14^{ARF}$ and ANRIL, which regulate proliferation, oncogenesis, senescence and ageing
- $p16^{INK4A}$ mediates an ageing-related decline in beta cell proliferation in mice, and may regulate insulin secretion independently of beta cell mass
- *CDKN2A/B* and related genes also regulate adipocyte differentiation, inflammation, hepatic insulin clearance, fasted–fed transition and muscle metabolism
- Polymorphisms at *CDKN2A/B* have not yet been clearly linked to local gene expression in any metabolic tissue
- The mechanisms linking the *CDKN2A/B* locus to type 2 diabetes risk in human populations remain unknown

The *CDKN2A/B* locus impacts human diabetes risk

The *CDKN2A/B* locus, at chromosome 9p21, influences diabetes risk across varied ethnicities and geography [1–3], including people of European [4–9], Asian [10–24], Indian [25], Pakistani [26], Mexican [27–29] and Arab [30–32] descent. *CDKN2A/B* polymorphisms impact risk of gestational diabetes [33–35], early progression to type 2 diabetes after gestational diabetes [36], post-transplant diabetes [37, 38] and cystic fibrosis-related diabetes [39]. *CDKN2A/B* is not associated with type 1 diabetes risk [40–42], but is associated with rapid decline in beta cell function [43], and progression to diabetic nephropathy, in individuals with type 1 diabetes [44]. *CDKN2A/B* polymorphisms contribute only a fraction of observed heritable type 2 diabetes risk [2]. How *CDKN2A/B* influences diabetes risk remains uncertain [1–3].

***CDKN2A/B* is a hotspot influencing genetic risk for many diseases** The *CDKN2A/B* locus also influences risk for vascular conditions [1], including coronary artery disease [45–49], aneurysm and ischaemic stroke [50, 51], as well as glaucoma [52, 53], Alzheimer's disease [54], endometriosis [55], periodontitis [56], ageing-related diseases [3, 57] and numerous cancers [58]. Intriguingly, the region of *CDKN2A/B* influencing type 2 diabetes risk is physically separated from regions contributing risk for other diseases, even for type 2 diabetes-related disorders such as cancer and cardiovascular disease [59–61].

General biology of *CDKN2A/B* locus genes

In order to consider how single nucleotide polymorphisms (SNPs) in the *CDKN2A/B* region may impact type 2 diabetes risk, an important starting point is the known biology of local genes.

The *CDKN2A/B* locus encodes three coding transcripts and a long non-coding RNA The human *CDKN2A/B* locus (Fig. 1) encodes three proteins: p14 alternate reading frame ($p14^{ARF}$) ($p19^{ARF}$ in mice), p15 and p16 inhibitors of cyclin dependent kinase 4 ($p15^{INK4B}$ and $p16^{INK4A}$), and a long non-coding RNA (lncRNA) called ANRIL (also known as CDKN2B-AS) [1, 62, 63]. $p16^{INK4A}$ and $p14^{ARF}$, encoded by the *CDKN2A* gene, share common second and third exons but have different first exons and promoters about 20 kb apart [64]. $p16^{INK4A}$ and $p14^{ARF}$ are in alternate reading frames, resulting in unrelated peptide sequences despite the common mRNA sequence. *CDKN2B*, about 30 kb from *CDKN2A*, encodes $p15^{INK4B}$ [65, 66]. Smaller splice variants of both $p16^{INK4A}$ (p12) and $p15^{INK4B}$ (p10) have been described [64, 67, 68]. *ANRIL*, which overlaps the $p14^{ARF}$ promoter and two exons of $p15^{INK4B}$ [69], transcribed by RNA polymerase II [70] in the antisense direction to *CDKN2B*, is spliced into linear or circular isoforms [69, 71, 72]. The mouse *Cdkn2a/b* locus, on chromosome 4, encodes $p16^{INK4A}$, $p19^{ARF}$ and $p15^{INK4B}$ in a similar arrangement to the human locus, but with a different lncRNA called *AK148321* in a position similar to *ANRIL* [73].

$p14^{ARF}$, $p15^{INK4B}$ and $p16^{INK4A}$ are cell cycle inhibitors *CDKN2A/B* proteins block cell cycle progression and influence tumorigenesis, senescence and ageing [62, 63]. $p16^{INK4A}$ and $p15^{INK4B}$ are cyclin-dependent kinase (CDK) inhibitors that prevent activation of CDK4/6 by D-cyclins (Fig. 2). CDK4/6 phosphorylates retinoblastoma (Rb); hypophosphorylated Rb represses early region 2 transcription factor (E2F) to prevent cell cycle entry [74]. $p14^{ARF}$, also anti-proliferative, acts by stabilising the tumour suppressor p53 by sequestering its negative regulator, mouse double minute 2

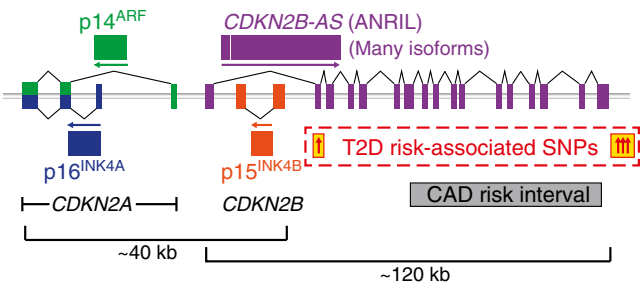

Fig. 1 The human *CDKN2A/B* locus at 9p21 contains genes encoding $p16^{INK4A}$, $p14^{ARF}$, $p15^{INK4B}$ and the lncRNA *ANRIL*. *CDKN2A* encodes both $p16^{INK4A}$ and $p14^{ARF}$, which share exons 2 and 3 but in different reading frames, producing unrelated peptides. Polymorphisms influencing type 2 diabetes risk are physically separate from the coronary artery disease risk interval. Diagram not to scale. CAD, coronary artery disease; T2D, type 2 diabetes

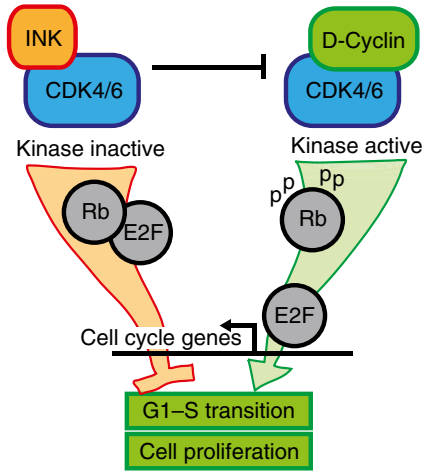

Fig. 2 INK family inhibitors ($p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$ and $p19^{INK4D}$) bind CDK4/6 and prevent CDK binding and activation by D-cyclins. Hypophosphorylated Rb sequesters E2Fs; active (cyclin-bound) CDK phosphorylates Rb, de-repressing transcriptional activity of activating E2Fs to promote cell cycle entry

homologue (MDM2) [75]. p53 reduces cell cycle entry through the CDK-interacting protein/kinase inhibitory protein (CIP/KIP) family inhibitor p21. The p10 and p12 splice variants of $p16^{INK4A}$ and $p15^{INK4B}$ also inhibit the cell cycle, but through slightly different mechanisms [64, 67]. ANRIL has widespread influences on gene expression, and impacts the cell cycle by regulating the expression of $p14^{ARF}$, $p15^{INK4B}$ and $p16^{INK4A}$ [73].

***CDKN2A/B* and tumorigenesis, senescence and ageing** The *CDKN2A/B* locus is inactivated by deletion, methylation or mutation in many cancers; *CDKN2A/B* aberrations correlate with advanced tumour stage and reduced overall and disease-free survival [58, 76]. Conversely, restoring $p16^{INK4A}$ suppresses tumour growth [77]. *CDKN2A* was the first familial melanoma gene identified [78]; germline loss-of-function *CDKN2A* mutations are the most frequent genetic events underlying familial melanoma susceptibility [79]. Activating *CDK4* mutations (*R24C* and *R24H*) that prevent inhibition by $p16^{INK4A}$ increase melanoma risk [80]. Intriguingly, loss of $p16^{INK4A}$ also contributes to pancreatic neuroendocrine tumours, which include islet tumours such as insulinomas [81–85]. Paradoxically, when not deleted, $p16^{INK4A}$ may be overexpressed in tumours and transformed cells [86, 87]. $p14^{ARF}$ loss is oncogenic as well, via MDM2/p53 and other mechanisms [62]. $p15^{INK4B}$ may be co-deleted with $p16^{INK4A}$ in haematological malignancies [62], and plays a role in TGF-β-induced pancreatic cancer [88, 89]. ANRIL is also implicated in many cancer pathways, downstream of oncogenic Ras, phospholipase D and specificity protein 1 (SP1), and upstream of Kruppel-like factor 2, p21 and CDKN2A/2B [90–93].

$p16^{INK4A}$ is an effector of senescence, an irreversible growth arrest that occurs when a cell reaches the end of its replicative lifespan [63, 94]. $p16^{INK4A}$ expression increases with age [62], triggered by ageing-dependent gene demethylation, telomere shortening and other senescence inducers such as oncogenic activation, oxidative stress, nutrient deprivation and DNA damage [95, 96]. Mice and humans with the R24C activating mutation of CDK4 are resistant to senescence [97]. A recent study has shown that ablating senescent cells, as defined by $p16^{INK4A}$ expression, extends longevity and forestalls ageing-related tissue functional decline [98]. $p14^{ARF}$ is also linked to senescence, independent of $p16^{INK4A}$ [99].

Evidence that *CDKN2A/B* influences type 2 diabetes risk via beta cell mass and proliferation

The best-known role of *CDKN2A/B* gene products in a metabolic tissue is the ageing-related negative impact of $p16^{INK4A}$ on beta cell proliferation and regeneration [100, 101]. The critical dependence of mouse beta cell mass on CDK4 further supports the importance of this pathway in islet biology. Here, we will review the known roles of *CDKN2A/B* genes in islets.

Effects of *CDKN2A/B*-related proteins on metabolic tissues

Tissue	Protein	Effects
Islets	$p16^{INK4A}$	Restricts beta cell proliferation in ageing, restricts beta cell regeneration, mediates overnutrition-related senescence, reduces insulin secretory function
	CDK4	Required for postnatal beta cell mass expansion
	Cyclin D1, D2	Required for postnatal beta cell mass expansion
Adipose	$p15^{INK4B}$	Inhibits adipocyte differentiation
	$p16^{INK4A}$	Modulates adipose macrophage activation and polarisation
	CDK4	Promotes adipocyte differentiation
Liver	$p16^{INK4A}$	Restrains hepatic gluconeogenesis
	Cyclin D1/CDK4	Regulates fasted–fed transition
Muscle	CDK4	Impacts mitochondrial oxidative metabolism via E2F1

Beta cell proliferation decreases with age Type 2 diabetes incidence increases with age, related to declining beta cell proliferation, beta cell function and/or insulin sensitivity [102–105]. Human autopsy studies report an age-dependent reduction in beta cell proliferation [102, 106, 107]. The importance of this has been contested on the basis that proliferation is rare even in young humans, such that a loss with ageing is not meaningful [108]. The demonstration that some measures of proliferation on autopsy specimens do not accurately reflect in vivo proliferation raises questions about the validity of postmortem analyses [109]. Beta cell mass is reported to be unchanged, or only modestly reduced, in ageing Japanese [108, 110]. As human beta cells may be extremely long lived, maintenance of beta cell mass may not require much ongoing proliferation [103]. Adult beta cell mass is thought to depend on early postnatal beta cell proliferation [106, 107]. In rodents, an age-associated decline in proliferation is clear [105]. Intriguingly, old islets exposed to young circulation via parabiosis or transplantation recovered a youthful proliferation frequency, suggesting the loss of proliferation is due to a circulating factor [111]. However, the rate of human beta cell proliferation is lower than that of rodents, and engrafting human islets into mice does not increase human beta cell proliferation to the rodent frequency [112, 113].

$p16^{INK4A}$ expression in rodent and human islets increases with age Given the low beta cell replication frequency, cell cycle inhibitors have been a focus in this field. $p16^{INK4A}$ is expressed in mouse islets [101, 114–126]. Telomere shortening impacts islet $p16^{INK4A}$ expression as it does in other tissues [121]. The abundance of $p16^{INK4A}$ in young healthy islets is low, however, at or below the limit of detection in our hands by quantitative PCR using primers that do not amplify $p14^{ARF}$ (data not shown). Mouse islet $p16^{INK4A}$ abundance increases with ageing [101, 116, 117, 119, 121, 124–126]. $p16^{INK4A}$ is expressed in human islets [110, 127–132] in an age-dependent fashion [128], possibly related to progressive *CDKN2A* locus demethylation with age [96]. In human pancreas, nuclear $p16^{INK4A}$ staining was occasionally present in fetal beta cells, but was evident in 63% of adult beta cells [127]; $p16^{INK4A}$ staining was significantly lower in younger (age 0–9 years) than older (age 10–59 and 60–79 years) samples [110].

Regulation of islet $p16^{INK4A}$ expression and subcellular localisation Islet $p16^{INK4A}$ expression is regulated at the RNA level via epigenetic histone modification by the polycomb repressors enhancer of zeste 2 (EZH2) and B cell-specific Moloney murine leukaemia virus insertion site 1 (BMI1). BMI1 and EZH2 bind the $p16^{INK4A}$ promoter to increase H3K27 trimethylation and reduce histone 2A (H2A) ubiquitination and H3K4 trimethylation via trithorax group (TrxG) mixed lineage leukaemia protein 1 (MLL1) [116, 119]. BMI1 and EZH2 occupancy of the $p16^{INK4A}$ promoter is reduced with ageing, resulting in de-repression of gene expression. Knockdown of EZH2 reduced BMI1 presence at the $p16^{INK4A}$ promoter, suggesting cooperative recruitment [116]. Overexpression of EZH2 suppressed $p16^{INK4A}$ expression in young but not old mice; in aged islets, MLL1 prevented EZH2 recruitment to the $p16^{INK4A}$ promoter [133]. EZH2 expression in human islets is inversely related to age [119]. These observations may apply to $p16^{INK4A}$ and $p14^{ARF}$, but not $p15^{INK4B}$ [119].

Insulin signalling pathways impact islet $p16^{INK4A}$ expression. BMI1 is regulated by p38 mitogen-activated protein kinase (p38MAPK). Mice expressing an activation-resistant allele of *p38Mapk* (also known as *Mapk14*) had reduced abundance of $p15^{INK4B}$, $p16^{INK4A}$ and $p14^{ARF}$ in aged islets; age-dependent BMI1 repressor loss at the $p16^{INK4A}$ promoter was reduced, possibly through p38MAPK target MAPK-activated protein kinase 3 (MK3) phosphorylation of BMI1 [134]. The age dependence of p38 activation was due to reduction of the wild-type p53-induced phosphatase 1 (WIP1) p38 phosphatase with age. Phosphoinositide 3-kinase (PI3K) signalling also influences $p16^{INK4A}$ expression; mice with phosphatase and tensin homologue (PTEN) deficiency had increased $p16^{INK4A}$ expression via a pathway involving cyclin D1, E2Fs and EZH2 [124, 125].

The protein subcellular localisation of cell cycle regulators likely impacts activity. In human beta cells, directly driving the cell cycle by overexpressing cyclin D3 and CDK6 caused $p16^{INK4A}$, but not $p15^{INK4B}$, to shift from the cytoplasm to the nucleus [130]. Nuclear relocalisation of $p16^{INK4A}$ depended on culture duration; at shorter time points after transduction (24–48 h) $p16^{INK4A}$ was less likely to be nuclear than in control cells, but after 72 h $p16^{INK4A}$ was more likely to be nuclear. The proportion of beta cells with nuclear $p16^{INK4A}$ expression increased with culture duration in control islets as well. Human islet cells undergo growth arrest after 10–15 divisions, with shortened telomeres and increased $p16^{INK4A}$ expression [135]. Taken together, this suggests that driving the cell cycle by activating CDK6 in human beta cells may hasten culture-related senescence.

$p16^{INK4A}$ mediates ageing-related loss in beta cell proliferation Overexpression of $p16^{INK4A}$ reduced beta cell proliferation in young mice, and deletion of $p16^{INK4A}$ rescued the age-related loss of proliferation [101]. Mice lacking BMI1 or EZH2, such that $p16^{INK4A}$ is prematurely induced, have reduced beta cell proliferation; in the case of EZH2, dependence of proliferation loss on $p16^{INK4A}$ was confirmed [117, 119]. Overexpression of EZH2 repressed $p16^{INK4A}$ and increased proliferation in young mice; in older mice the same effects were seen but only if MLL1 was also reduced [133]. Ex vivo, knockdown of $p16^{INK4A}$ using small interfering (si)RNA rescued the loss of proliferation induced by NEFA exposure [123]. Alterations in the WIP1/p38MAPK/BMI1 or

PTEN/E2F/EZH2 pathways that reduced $p16^{INK4A}$ expression increased beta cell proliferation in ageing mice [124, 134]. Loss of $p16^{INK4A}$ and a related INK family inhibitor, $p18^{INK4c}$, synergistically increased beta cell proliferation in a CDK4-dependent manner [136]. $p15^{INK4B}$ also impacts beta cell proliferation; transgenic overexpression of TGF-β in alpha cells resulted in pancreas and islet hypoplasia associated with increased islet expression of $p15^{INK4B}$ [137].

$p16^{INK4A}$ restricts beta cell regeneration The impact of ageing on beta cell regeneration is an area of controversy. Beta cell proliferation after partial pancreatectomy, exendin-4 treatment, high-fat feeding or streptozotocin ablation is lower in ageing mice than in young controls [101, 104, 117]. On the other hand, after diphtheria toxin ablation or glucokinase activation mice even beyond 2 years of age retained capacity for regenerative proliferation [138]. $p16^{INK4A}$ mediates age-related loss of islet regenerative capacity [101]. Loss of BMI1 and EZH2 impair beta cell proliferation in response to regenerative stimuli [117, 119]. BMI1 may also promote exocrine pancreas regeneration [139, 140]. A synthetic antagonist of hepatocyte nuclear factor (HNF)4α increased beta cell proliferation by suppressing the expression of CDK inhibitors including $p16^{INK4A}$; inhibiting HNF4α induced alpha, beta and delta cell proliferation after beta cell ablation [141]. Beta cell proliferation in response to parathyroid hormone-related peptide (PTHrP) was associated with decreased expression of $p16^{INK4A}$ [122]. On the other hand, increased islet expression of $p16^{INK4A}$ may have prevented the expected increase in beta cell proliferation with overexpression of activated S6 kinase [120].

CDK4: critical for postnatal mouse beta cell mass INK family inhibitors, which include $p15^{INK4B}$ and $p16^{INK4A}$ as well as $p18^{INK4c}$ and $p19^{INK4d}$, bind to and inhibit CDK4/6 (Fig. 2) [74]. The essential role for CDK4 in mouse islet biology supports the importance of INK family inhibitors in islets. Mice lacking CDK4 develop severe insulin-deficient diabetes because of hypoplastic islets [142]. Intriguingly, CDK4 deletion also impacts other endocrine systems: male and female infertility, and poor growth, are possibly related to pituitary defects. Islet morphology is normal at birth, suggesting CDK4 is not required for pancreatic development, but islets fail to expand during postnatal growth. Conversely, mice expressing the INK-resistant mutant CDK4 'R24C' have hyperplastic islets due to increased postnatal beta cell proliferation [142, 143]. Mutation of the arginine at CDK4 position 24 is oncogenic in many tissues, despite also having impaired binding to D-cyclins and reduced efficacy as an Rb kinase [144]. Rodent insulinoma cell lines have increased expression of CDKs including 4 and 6; overexpressing CDK4/6 in rat islets increased proliferation [86].

D-cyclins and mouse beta cell proliferation Cyclins D1, D2 and D3 activate CDK4/6 kinases, linking external growth signals with cell cycle regulation. INK family inhibitors bind CDKs near the D-cyclin binding site, preventing D-cyclin binding and kinase activation. Consistent with the importance of CDK4 in islets, D-cyclins also regulate beta cell proliferation in mice. Cyclin D2 and, to a lesser extent, cyclin D1, are required for postnatal beta cell proliferation [145, 146]. Like CDK4, D-cyclins are not required for prenatal pancreatic development. Upstream signalling events that promote beta cell proliferation, such as nutrient excess, insulin signalling and Wnt signalling, increase D-cyclin abundance in islets [122, 123, 147–155]. Islet overexpression of a stabilised cyclin D2 increased islet mass, although proliferation was not increased at the time points tested [151].

Metabolic insults may cause premature islet ageing Metabolic stress influences ageing-related markers in general, and $p16^{INK4A}$ expression specifically. Failed human islet grafts in diabetic nude mice showed widespread $p16^{INK4A}$ immunostaining [129]. Energy restriction, an intervention that reduces metabolic load and delays ageing, decreased ageing-related $p16^{INK4A}$ expression in many tissues [126]. On the other hand, intrauterine protein-energy malnutrition increases type 2 diabetes risk. In rats, in utero nutrient deprivation reduced postnatal beta cell proliferation and led to premature ageing, with telomere shortening and induction of senescence markers including $p16^{INK4A}$ [156]. Maternal islets are also sensitive; postpartum, but not never-pregnant, female mice treated with the endocrine disruptor bisphenol-A developed glucose intolerance related to lost beta cell mass and insulin secretory function, with increased $p16^{INK4A}$ and p53 and reduced cyclin D2 and CDK4 [118].

The impact of lipid excess on beta cell proliferation remains controversial [123, 157, 158]. In vivo or ex vivo exposure to glucolipotoxicity reduced proliferation and induced islet $p16^{INK4A}$ expression in young mice [123]. Senescence markers in islets heralded metabolic decompensation after long-term high-fat feeding [159]. In contrast, short-term intravenous infusion with glucose and lipid in rats did not induce $p16^{INK4A}$ expression under conditions where beta cell proliferation was high [158]. Nutrition deprivation followed by nutrient excess induced islet senescence markers in rats [156]. The impact of nutrients on islet $p16^{INK4A}$ expression and senescence may depend on other factors related to development, genetics, duration of insult, overall stress load and stress tolerance.

Differences between cell cycle regulation in mouse and human beta cells: do D-cyclins, CDK4/6 or $p16^{INK4A}$ influence human beta cell proliferation? Surveys of cell cycle regulator expression report similar patterns for rodent and human islets; of 34 proteins tested, only CDK6, E2F3,

E2F7 and E2F2 show differences, with CDK6, E2F3 and E2F7 present in human islets but absent in mouse islets, and the converse for E2F2 [132, 160, 161]. Some reports suggest cyclin D2 may be less abundant than D1 and D3 in human islets [128, 132, 162]; however, recent reports link the *CCND2* genomic locus, which encodes cyclin D2, with human type 2 diabetes risk [163–165]. A low-frequency non-coding variant at *CCND2* reduced type 2 diabetes risk by about half in European populations; risk reduction was due to increased insulin secretion [164]. The impact of *CCND2* was sex dependent, with a larger effect in men than women [166]. In ex vivo human islet cultures D-cyclins and CDK4/6 were detected in the cytoplasm rather than the nuclear compartment [131]; the same was true in rat islet cells for those cell cycle activators tested (cyclins D1/D2 and CDK2) [130]. Overexpression of D-cyclins was not as effective as CDKs in increasing human beta cell proliferation, this was perhaps related to the cytoplasmic location of CDKs, the abundance of INK family inhibitors [131, 162] or synergy with nutrient signals [167]. Data linking $p16^{INK4A}$ to reduced proliferation in human beta cells are somewhat limited. In autopsy material, $p16^{INK4A}$ was never detected in actively proliferating beta cells [127], and $p16^{INK4A}$ staining inversely correlated with beta cell mass [110]. Ex vivo human beta cells with nuclear $p16^{INK4A}$ after overexpression of cyclin D3 and CDK6 did not stain for the proliferation marker Ki67, despite 10–20% of beta cells in these cultures staining for Ki67 [130]. Knockdown of $p16^{INK4A}$ in the transformed EndoC-bH1 human beta cell line did not impact proliferation; however, transformation bypasses normal cell cycle regulation [168].

CDKN2A/B gene products may impact type 2 diabetes risk via other mechanisms

Evidence suggesting $p16^{INK4A}$ does not increase type 2 diabetes risk by reducing islet mass Several observations contradict the concept that $p16^{INK4A}$ impacts diabetes incidence via effects on beta cell mass. The combined loss of $p16^{INK4A}$ and $p14^{ARF}$ was not sufficient to restore the glucose induction of mitotic genes lost in aged islets [169]. Old islets that recovered proliferation when exposed to young mouse circulation did not have reduced $p16^{INK4A}$ expression [111]. Perhaps most puzzling from an islet-centric viewpoint are observations from the Super-Ink4/ARF mouse, which contains an extra copy of the entire *Cdkn2a/b* locus [170]. Despite modestly increased expression of $p15^{INK4B}$, $p16^{INK4A}$ and $p14^{ARF}$ in several tissues, these mice have improved glucose tolerance with ageing from enhanced insulin sensitivity in liver and muscle. Beta cell proliferation and islet number are unaffected. Reduced insulin secretion is observed, but is likely to be secondary to the improved insulin sensitivity. Also curious, a survey of islet gene expression at type 2 diabetes-linked loci in diabetes-prone New Zealand Obese (NZO) mice vs diabetes-resistant *ob*/*ob* mice revealed increased $p15^{INK4B}$ and $p16^{INK4A}$ expression in the diabetes-resistant mice [115].

$p16^{INK4A}$ may influence insulin secretory function, insulin clearance and insulin sensitivity Some observations suggest an impact on insulin secretory function independent of the effects on beta cell mass (see the text box 'Effects of *CDKN2A/B*-related proteins on metabolic tissues'). Knockdown of $p16^{INK4A}$ in the EndoC-bH1 human beta cell line increased insulin secretion [168]. In mice, haploinsufficiency for telomerase increased $p16^{INK4A}$ expression and impaired insulin secretion via altered regulation of exocytosis. These mice were glucose intolerant even though beta cell mass was normal, suggesting a primary effect on beta cell function rather than proliferation [121]. CDK4 also regulates insulin secretion, through Rb-dependent transcriptional regulation of potassium inward rectifying channel 6.2 (Kir6.2) [171]. In human studies, individuals from familial melanoma kindreds with heterozygous loss of function of *CDKN2A* had increased insulin secretion, impaired insulin sensitivity and reduced hepatic insulin clearance [168].

***CDKN2A/B* locus genes impact adipose, liver and muscle** *CDKN2A/B* locus genes and their CDK targets influence adipose, liver and muscle biology. Deletion of a region of mouse chromosome 4, orthologous to the human 9p21 cardiovascular disease risk interval, reduced local gene expression and increased body weight, linking the region with obesity and metabolic risk [172]. ANRIL regulates genes involved in glucose and fatty acid metabolism [173]. $p15^{INK4B}$ is highly expressed in subcutaneous adipose tissue and may inhibit expandability of the subcutaneous fat depot, a critical protection against overnutrition toxicity [174, 175]. Mice lacking CDK4 in all tissues are smaller, with less fat mass, and have insulin resistance in addition to insulin deficiency [176]. CDK4 regulates adipogenesis, via both Rb-dependent and Rb-independent mechanisms, including direct phosphorylation of insulin signalling intermediates [177–179]. *CDKN2A/B* locus genes also impact adipose tissue inflammation; $p16^{INK4A}$ modulates the activation and polarisation of adipose-associated macrophages [180], although deleting $p16^{INK4A}$ in bone marrow-derived cells did not impact glucose metabolism, even under obese conditions [181]. In liver, $p16^{INK4A}$ regulates hepatic gluconeogenesis independently of the cell cycle [182]. $p16^{INK4A}$ deficiency increased liver glucose production via a protein kinase A (PKA)-mediated induction of gluconeogenic gene expression. CDK4 participates in the hepatocyte fasting–fed transition by phosphorylating and activating the general control of amino acid synthesis protein 5-like 2 (GCN5) histone acetyltransferase, which regulates PPARG coactivator 1 α (PGC-1A) and hepatic glucose metabolism [183]. CDK4 also impacts muscle mitochondrial

oxidative metabolism [184]. These observations, collectively, greatly broaden the potential mechanism(s) by which *CDKN2A/B* polymorphisms might impact diabetes risk.

How might *CDKN2A/B* polymorphisms influence type 2 diabetes risk?

Type 2 diabetes SNPs at *CDKN2A/B* are non-coding, and high-resolution mapping of the *CDKN2A/B* locus did not reveal SNPs with greater disease association than known SNPs [185]. Attention has thus turned to the regulation of local genes.

Do *CDKN2A/B* polymorphisms influence local gene expression? Testing whether genomic polymorphisms alter mRNA abundance is performed by expression quantitative trait locus (eQTL) analysis. Thus far, however, eQTL analysis for the *CDKN2A/B* locus has failed to produce major mechanistic breakthroughs. Type 2 diabetes SNPs at *CDKN2A/B* were not associated with expression of *CDKN2A* or *CDKN2B* in pancreas, liver or colon [186], nor in pancreatic islets themselves [187]. *CDKN2A/B* SNPs impacting cardiovascular risk, outside the type 2 diabetes region, influence ANRIL expression [71, 188]. Both type 2 diabetes and non-type 2 diabetes SNPs at this locus appear to have a stronger effect on ANRIL expression than *CDKN2A* or *CDKN2B* expression [189]. Expression of ANRIL is reported to correlate with expression of *CDKN2A* and *CDKN2B* in many tissues, suggesting coordinated local regulation [69, 71, 76, 189], although independent regulation is also reported [76, 126]. Deletion of a large region including the type 2 diabetes-risk locus plus part of *ANRIL* reduced *CDKN2A* and *CDKN2B* expression in several vascular-relevant cell types in mice [172]. ANRIL regulates gene expression at the *CDKN2A/B* locus by recruiting polycomb proteins chromobox homologue 7 (CBX7 [PRC1]) and suppressor of zeste 12 (SUZ12 [PRC2]) to modulate epigenetic repression by H3K27 methylation [70, 72, 73, 90, 190]. ANRIL can also regulate distant genes with retrotransposon Alu repeats in their promoters [191]. In sum, existing eQTL analyses do not identify a mechanism by which polymorphisms at *CDKN2A/B* influence type 2 diabetes risk through local gene expression, but studies may have been performed in the wrong cell type, developmental, environmental or nutritional state to identify the point of activity. The requirement to perform these studies in human tissues, the difficulty in obtaining human samples, and caveats introduced through postmortem state, tissue collection and ex vivo culture are barriers to this type of study.

***CDKN2A/B* polymorphisms are located in regulatory enhancers** *Cis*-regulatory elements are found near the type 2 diabetes-risk interval [192–194]. Disease-associated SNPs are predicted to disrupt transcription factor binding sites, including some factors with known roles in beta cell development or survival, including, v-maf avian musculoaponeurotic fibrosarcoma oncogene homologue B (MAFB), NK homeobox protein 6.1 (NKX6.1), nuclear factor of activated T lymphocytes (NFAT), FOXA2, forkhead box A2 (FOXA2), nuclear factor κβ (NFκB), hepatocyte nuclear factor 1 (HNF1) and CCAAT-enhancer-binding protein homologous protein (CHOP) [90, 192, 194]. Polymorphisms may also impact microRNA regulation of transcription or translation [195], although most SNPs at the type 2 diabetes locus are non-coding. An enhancer identified in tumours, $RD^{INK4/ARF}$, interacts with oncoproteins to silence the *CDKN2A/B* locus [196, 197]. Deletions of $RD^{INK4/ARF}$ have been detected in pancreatic neuroendocrine tumours, implying activity in islet cells [198].

Do polymorphisms influence epigenetic regulation at the *CDKN2A/B* locus? Epigenetic modification regulates gene expression at this locus in islets [116, 119]. The type 2 diabetes-associated rs564398 removes a DNA methylation CpG site, reducing methylation of other local CpG sites and decreasing insulin content in human islets, although without impacting local gene expression [199]. Histone modifications can be influenced by polymorphisms as well; many epigenetic modifiers act at the *CDKN2A/B* locus [200]. Metabolic inputs, such as overfeeding or energy restriction, influence CpG methylation and histone modification at *CDKN2A/B* in humans [201, 202]. Whether this is influenced by type 2 diabetes-risk polymorphisms remains unknown. Taken together, although many mechanisms exist by which polymorphisms at *CDKN2A/B* might influence local gene expression, none has yet been proved.

Evidence that human *CDKN2A/B*-related type 2 diabetes risk involves beta cells

***CDKN2A/B* genotype influences insulin secretory capacity** Many type 2 diabetes risk polymorphisms, including at *CDKN2A/B*, may impact human diabetes risk by altering insulin secretory capacity [203]. Age influences the association between *CDKN2A/B* polymorphism rs10811661 and type 2 diabetes, consistent with the known interaction between age and $p16^{INK4A}$ activity in islets [204]. The rs10811661 'T' (risk) allele is associated with reduced insulin secretion after both oral and intravenous glucose challenge [205, 206]. In Europeans undergoing hyperglycaemic clamp, although *CDKN2A/B* was not independently associated with beta cell function, a composite including *CDKN2A/B* among eight loci predicted reduced first-phase insulin secretion [207]. In this cohort, each additional risk allele lowered glucose-stimulated insulin secretion (GSIS) by 5%, but no effect was seen on insulin sensitivity. The detrimental effect on insulin secretion

was of similar magnitude in individuals with normal glucose tolerance and impaired glucose tolerance, suggesting *CDNK2A/B* impact pre-dates metabolic decompensation. In a large meta-analysis including more than 58,000 individuals, *CDKN2A/B* was associated with insulinogenic index, acute insulin response, fasting glucose or HOMA-B, but not with insulin sensitivity index, fasting insulin, fasting proinsulin, or HOMA-IR [208]. A parallel study of whether *CDKN2A/B* genotype impacts islet function in vivo and ex vivo revealed an in vivo association with reduced insulin secretion and disposition index, but not insulin sensitivity or glucagon secretion, but no striking impact on ex vivo function [209]. Individuals with reduced $p16^{INK4A}$ or $p16^{INK4A}/p14^{ARF}$ activity have increased basal and stimulated insulin secretion [168].

Little evidence for or against a role in *CDKN2A/B* in human beta cell mass accrual The observed reduction in insulin secretory capacity with *CDKN2A/B* polymorphisms could be due to reduced beta cell function (glucose sensing, insulin production, stimulus-secretion coupling) or reduced beta cell mass. Despite the data linking $p16^{INK4A}$ to beta cell mass in mice, few studies have assessed whether *CDKN2A/B* genotype impacts human beta cell proliferation or mass. A limitation is that human beta cell mass and proliferation cannot easily be measured in living people. In a single study, *CDKN2A/B* polymorphisms were not associated with AIR^{max}, a surrogate for beta cell mass [207]. On the other hand, the contribution of $p16^{INK4A}$ loss to pancreatic neuroendocrine tumour risk may imply a role in human islet cell proliferation [81–85].

Evidence that *CDKN2A/B* influences type 2 diabetes risk via non-islet mechanisms

Evidence that *CDKN2A/B* increases diabetes risk without impacting islet function *CDKN2A/B* was not associated with proinsulin conversion to insulin [210] or the effect of ambient glycaemia on insulin secretion [211]. Although *CDKN2A/B* significantly impacted risk of diabetes in a Han Chinese population, this was not related to reduced HOMA-B, although other loci (*CDKAL1*, *IGF2BP2* and *SLC30A8*) did impact beta cell function in this population [212]. In a relatively young cohort of European individuals tested by OGTT, *CDKN2A/B* was not associated with insulin secretion or glucose sensitivity, although *CDKAL1* and *HHEX* were [212]; intriguingly, in this cohort two of three *CDKN2A/B* risk alleles tested (rs10757283 and rs564398 but not rs10811661) showed a trend towards reduced insulin sensitivity. In a hyperglycaemic clamp study, *CDKAL1* and *IGF2BP2*, but not *CDKN2A/B*, were associated with reduced first-phase insulin secretion [213]. Complicating matters, the influence of *CDKN2A/B* type 2 diabetes polymorphisms on insulin secretion may depend on ambient insulin sensitivity [214].

***CDKN2A/B* impacts beta cell response to particular diabetes therapies** In a post-hoc analysis of the Diabetes Prevention Program (DPP), *CDKN2A/B* identity was not related to baseline insulin secretory capacity; however, unique among the eight loci tested, *CDKN2A/B* predicted response to therapy. Insulin secretion, but not insulin sensitivity, was improved in protective-allele carriers, but only in the thiazolidinedione treatment arm [215]. In another pharmacogenetics study, of 27 diabetes loci tested, only *CDKN2A/B* rs10811661 predicted response to therapy: the protective allele was associated with greater response to sulfonylurea therapy [216]. The *CDKN2A/B* locus may [217] or may not [218] influence the metabolic response to exercise.

Evidence that *CDKN2A/B* impacts diabetes risk through other metabolic tissues The intriguing relationship between *CDKN2A/B* genotype and response to thiazolidinedione in the DPP cohort suggests activity in adipose tissue; as lipids negatively impact pancreatic beta cell proliferation and function this may represent an adipocyte–beta cell axis [123, 157, 215]. In a Japanese population, *CDKN2A/B* was not related to visceral fat accumulation [219], but in an Indian sibling-pair study, *CDKN2A/B* polymorphisms impacted fasting insulin and HOMA-IR but not HOMA-B, suggesting a primary effect on insulin sensitivity [220]. The melanoma kindred study also found that individuals haploinsufficient for $p16^{INK4A}$ had impaired insulin sensitivity [168]. In the Helsinki Birth Cohort, *CDKN2A/B* type 2 diabetes-risk polymorphisms were associated with reduced birthweight, suggesting the possibility that *CDKN2A/B* diabetes risk might be related to developmental impact of this locus, bringing an additional temporal variable to play [221].

Type 2 diabetes CDKN2A/B polymorphisms are not yet clinically useful Initial attempts to use type 2 diabetes genome-wide association study (GWAS) information to predict disease risk, optimise therapeutic impact and estimate prognosis have been disappointing. Even combining risk alleles only marginally improves type 2 diabetes prediction over clinical factors [222–224]. As described above, some progress has been made using the *CDKN2A/B* genotype to predict response to therapy [215, 216]. Although *CDKN2A/B* polymorphisms are not generally associated with longevity [225], cardiovascular mortality was paradoxically reduced in individuals homozygous for the type 2 diabetes-risk allele at rs10811661 [226]. In sum, despite progress, genotype information at the *CDKN2A/B* locus does not yet have meaningful clinical implications for individual patients.

Future directions and unresolved questions

Great progress has been made in identifying roles played by *CDKN2A/B* gene products in islets and other metabolic tissues, mostly in rodents but also in humans. $p16^{INK4A}$ and related proteins are critical regulators of rodent beta cell mass, but whether human *CDKN2A/B* polymorphisms influence type 2 diabetes risk via islet biology remains uncertain. Gene regulation analyses have not yet proved a relationship between type 2 diabetes SNPs and local gene expression, but the analyses to date may not have been performed in the relevant tissue, developmental stage, metabolic milieu and/or human subpopulation. *CDKN2A/B* genes impact islet, adipose, muscle, liver and immune cell function, at stages ranging from in utero development to ageing. Human biological variation likely influences *CDKN2A/B* effects. The availability of human samples across tissues and stages is a serious limitation in this field. We are hopeful that, in the future, *CDKN2A/B* polymorphisms will improve diabetes understanding and inform clinical decisions.

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