



HMGA1 is a novel downstream nuclear target of the insulin receptor signaling pathway

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High-mobility group AT-hook 1 (HMGA1) protein is an important nuclear factor that activates gene transcription by binding to AT-rich sequences in the promoter region of DNA. We previously demonstrated that HMGA1 is a key regulator of the insulin receptor (*INSR*) gene and individuals with defects in HMGA1 have decreased *INSR* expression and increased susceptibility to type 2 diabetes mellitus. In addition, there is evidence that intracellular regulatory molecules that are employed by the *INSR* signaling system are involved in post-translational modifications of HMGA1, including protein phosphorylation. It is known that phosphorylation of HMGA1 reduces DNA-binding affinity and transcriptional activation. In the present study, we investigated whether activation of the *INSR* by insulin affected HMGA1 protein phosphorylation and its regulation of gene transcription. Collectively, our findings indicate that HMGA1 is a novel downstream target of the *INSR* signaling pathway, thus representing a new critical nuclear mediator of insulin action and function.

HMGA1 is a small basic protein that binds to adenine-thymine (A-T) rich regions of DNA and functions mainly as a dynamic regulator of chromatin structure and gene transcription¹. Although without intrinsic transcriptional activating activity, HMGA1 acts as an 'architectural' transcription factor that can transactivate promoters through mechanisms that facilitate the assembly and stability of a multicomponent enhancer complex, the so-called enhanceosome, that drives gene transcription in response to multiple extracellular and intracellular signals^{2,3}. Such signals may affect HMGA1 function by inducing changes in post-translational protein modifications (including methylation, acetylation and phosphorylation) that markedly influence HMGA1 ability to interact with DNA substrates, other proteins and chromatin^{2,4-7}. DNA-binding activity of HMGA1 is reduced by HMGA1 phosphorylation, whereas protein dephosphorylation increases HMGA1-DNA binding affinity^{8,9}, suggesting a mechanism in which variations in the protein's biological activity can be specifically produced through mechanisms of phosphorylation/dephosphorylation of HMGA1, leading to gene repression or activation, respectively.

Previous observations *in vitro* suggest that changes in HMGA1 protein phosphorylation may occur in a mechanism involving the intracellular regulatory molecules *INSR* substrate-1 (*IRS-1*) and phosphatidylinositol 3-kinase (*PI-3K*)¹⁰ that are critical intermediate steps in transmitting the signal from the *INSR*. On the other hand, a relationship between HMGA1 and the *INSR* signaling system has been demonstrated before, showing that HMGA1 is a key regulator of the expression of the *INSR*, a major component of the insulin signaling pathway^{11,12}. However, the precise role and function of HMGA1 phosphorylation in the *INSR* signaling system have not yet been addressed and more empirical evidence is needed to ascertain the biological significance of this HMGA1 post-translational modification and its regulation within this context. Based on our findings in the present work, we are currently proposing that HMGA1 (specifically the HMGA1a isoform) is a novel downstream target of the *INSR* signaling pathway, which may play an important role in the regulation of insulin signaling and action *in vivo*.

Results

Activation of *IGFBP-1* gene transcription by HMGA1 and its suppression by insulin. Insulin-like growth factor-binding protein-1 (*IGFBP-1*) is a major member of the superfamily of IGF binding proteins. Under



physiological circumstances (e.g., in response to food and exercise), IGFBP-1 seems to be an important determinant in regulating IGF-I bioavailability and bioactivity^{13,14}. By binding endogenous IGF-I during fasting, IGFBP-1 may serve to prevent the hypoglycemic effects of IGF-I, thus supporting a physiological role for IGFBP-1 in glucose counterregulation¹⁵. Insulin plays a major role in the regulation of IGFBP-1, rapidly suppressing its production by the liver at the level of gene transcription¹⁵. Instead, a positive role of HMGA1 in *IGFBP-1* gene expression has been postulated previously on the basis of experimental evidence showing that HMGA1 binds the *IGFBP-1* gene promoter¹⁶. Consistent with this latter possibility, we previously found that IGFBP-1 expression was considerably reduced in *Hmga1*-knockout mice¹⁷. To investigate whether a functional link could be established between the INSR signaling system and HMGA1, we first performed experiments to see if HMGA1 had a direct role in activating *IGFBP-1* gene transcription. As shown in reporter gene assays, overexpression of HMGA1 significantly increased *IGFBP-1*-luciferase (*IGFBP-1*-Luc) activity in cells of both human (HepG2) and mouse (Hepa1) origin, and this effect occurred in a dose-dependent manner (Fig. 1a and Supplementary Fig. S1). Consistent with this, endogenous IGFBP-1 mRNA was reduced in cells pretreated with siRNA targeting *HMGA1* (Fig. 1a and Supplementary Fig. S1), indicating that activation of the *IGFBP-1* gene requires HMGA1. These results were corroborated by chromatin immunoprecipitation (ChIP) coupled with qRT-PCR of ChIP-ed samples, showing that binding of HMGA1 to the endogenous *IGFBP-1* chromosomal locus was increased in living HepG2 cells naturally expressing HMGA1, and was considerably decreased in cells exposed to siRNA against *HMGA1* (Fig. 1b). A functional link between insulin and HMGA1, at this level, was substantiated by showing that insulin-mediated inhibition of IGFBP-1 protein production was abolished in HepG2 cells markedly depleted of HMGA1 (Fig. 1c), as well as in cells treated with distamycin A (Fig. 1d), a small molecule inhibitor of HMGA1 protein binding to DNA¹². Insulin *per se* had no effect on HMGA1 protein expression in HepG2 cells (Fig. 1c,d), in which inhibition of IGFBP-1 protein production by insulin paralleled closely the decrease in HMGA1 occupancy at the endogenous *IGFBP-1* locus (Fig. 1e,f).

INSR signaling and HMGA1 activity. The relevance of HMGA1 for the INSR signaling system was supported in studies *in vivo*, under physiological circumstances where endogenous insulin production can vary (e.g., in response to fed and fasting states). As detected by ChIP *in vivo* coupled to qRT-PCR, HMGA1-DNA interaction was disrupted in liver of insulin-injected mice (Fig. 2a). Similar results were confirmed in liver from normal mice with augmented insulin levels as obtained after meal ingestion. As shown in Fig. 2a, binding of HMGA1 to the *IGFBP-1* locus was increased in mice under physiological fasting conditions when nutrients are limited, insulin levels are decreased and the insulin signaling cascade (IRS-1/PI-3K/Akt) is abrogated. Conversely, HMGA1-DNA interaction promptly decreased after refeeding, when insulin levels increase and insulin signaling is reactivated (Fig. 2a).

To probe further the role of HMGA1 in this signaling pathway, we examined the effect of wortmannin, a potent and selective inhibitor of the PI-3K/Akt cascade¹⁸, on HMGA1-mediated stimulation of IGFBP-1 in liver from insulin treated mice. Systemic treatment with wortmannin had no effect on the expression of the protein kinase Akt in liver; however, it significantly inhibited the level of insulin-induced Akt phosphorylation (Fig. 2b). Treatment with wortmannin resulted in 50–55% decrease by comparison with immunoblotting intensity for phosphorylated Akt in liver treated with insulin and vehicle (Fig. 2b). As measured by ChIP *in vivo*, on whole liver, and subsequent qRT-PCR of ChIP-ed samples, as a consequence of the inactivation of PI-3K/Akt cascade, occupancy of the *IGFBP-1* promoter by HMGA1 was increased in wortmannin-treated mice

compared to mice injected with insulin (Fig. 2b). Thus, these data suggest that HMGA1 can elicit *in vivo* functional responses that are acutely regulated through the INSR signaling pathway, whose activation/deactivation state appears to be decisive in the control of HMGA1-DNA interaction and function. This conclusion was substantiated by the following additional experimental observations, which revealed that an inverse correlation between increased IRS-1 phosphorylation and decreased HMGA1 DNA-binding activity existed in liver from insulin-injected mice, as measured by immunoprecipitation/western blot (IP/WB) of cytoplasmic proteins and electrophoretic mobility shift assay (EMSA) of liver nuclear extracts, respectively (Fig. 2c). Accordingly, IGFBP-1 mRNA abundance was reduced in liver from insulin-treated animals, and this reduction paralleled the decrease in IGFBP-1 protein levels as detected by IP/WB from liver lysates (Fig. 2c). Inhibition of insulin signaling *in vivo*, using the pharmacological PI-3K inhibitor wortmannin, by increasing HMGA1 DNA-binding, partially reversed the inhibition of IGFBP-1 mRNA and protein expression by insulin (Fig. 2d), thus indicating that phosphorylation of HMGA1 represents a fundamental step in INSR signaling and that functional regulation of HMGA1 by phosphorylation/dephosphorylation may be important during acute (short-term) regulation of glucose homeostasis in response to both hormonal and nutritional changes. This conclusion was supported by studies in primary cultured hepatocytes, showing that repression by insulin of the gluconeogenic genes phosphoenolpyruvate carboxykinase (*PEPCK*) and glucose-6-phosphatase (*G6Pase*), as well as the *IGFBP-1* gene, was differentially affected in primary cultured cells from normal wild-type and *Hmga1*-null mice. As shown in Fig. 2e, mRNA expression of *PEPCK*, *G6Pase* and *IGFBP-1* was lower in primary hepatocytes from *Hmga1*-null mice than in untreated wild-type-derived cells; following insulin treatment, mRNA levels for these genes decreased by 50% in cells from control mice, whereas no changes were detected in cells from mutant animals, indicating that the regulation of HMGA1, by changing its phosphorylation, is a critical event mediating the insulin's effect on these genes and that insulin action on gluconeogenesis is at least in part mediated by HMGA1.

Insulin induced post-translational phosphorylation of HMGA1 and its dynamic interaction with DNA. Phosphorylation of HMGA1 and its relevance for the INSR signaling system was investigated in detail in studies of post-translational modifications and nuclear localization of HMGA1 both *in vitro* and *in vivo*, following insulin treatment. As measured by liquid chromatography-mass spectrometry (LC-MS), a significant early increase of the tri-phosphorylated HMGA1a isoform protein was detectable in HepG2 cells at 30 min after insulin addition (Fig. 3a). Specificity of the insulin-induced HMGA1a phosphorylation was substantiated by the observation that HMG1, an HMG protein not involved in INSR signaling, did not change its phosphorylation state during insulin treatment (Fig. 3b). Consistently with the assumption that reduced HMGA1-DNA interaction after meals may reflect the physiological increase in insulin secretion and insulin signaling, enhanced HMGA1a phosphorylation was confirmed also *in vivo*, in liver from insulin-injected mice (Fig. 3c). As for other chromatin proteins, the apparently small magnitude of insulin-induced HMGA1 protein phosphorylation, both in HepG2 cells and in mouse liver, is compatible with the activation of a signaling pathway impinging on selected factors positioned at the level of specific regulatory sequences. In this regard, phosphorylation of histone H3 at serine 10 or 28 in the induction of immediate-early genes downstream of the MAPK pathways, constitutes one of the most striking examples¹⁹. As determined by tryptic-peptide mapping of the phosphorylated protein and the relative extracted ion count (EIC) of the peptides, di- and tri-phosphorylation of HMGA1 occurred predominantly at the C-terminal peptide 88-106 (Supplementary Fig. S2), a region

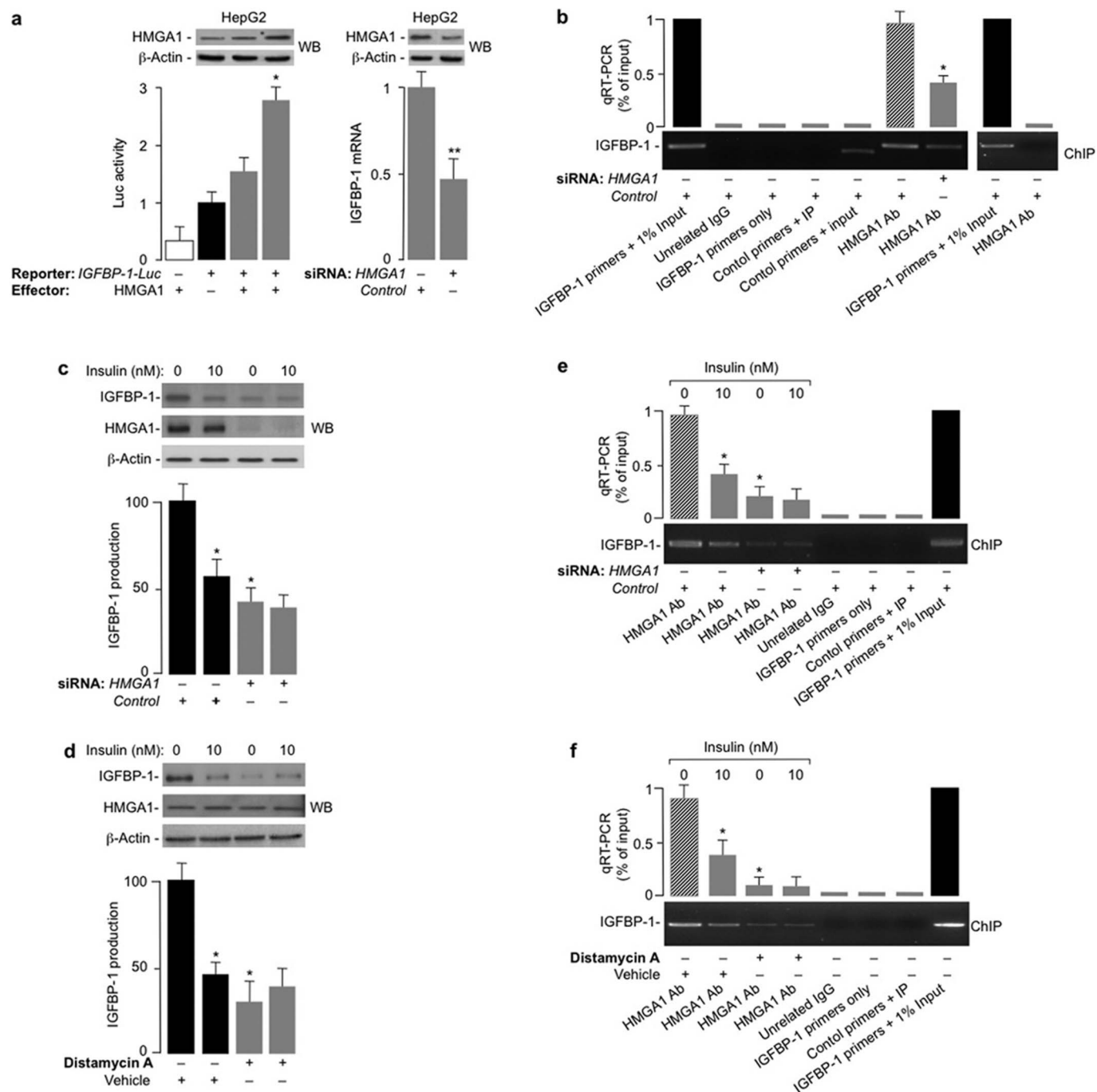


Figure 1 | Functional significance of HMGA1 for IGFBP-1 expression. (a) Left, human *IGFBP-1-Luc* reporter vector (2 μ g) was transfected into HepG2 cells, plus increasing amounts (0, 0.5 or 1 μ g) of *HMGA1* effector plasmid. Total plasmid DNA amounts were normalized with empty vector and Luc activity was assayed. Data represent the means \pm s.e.m. for three separate experiments; values are expressed as factors by which induced activity increased above the level of Luc activity obtained in transfections with *IGFBP-1-Luc* reporter vector plus the empty effector vector, which is assigned an arbitrary value of 1. White bar, pGL3-basic vector (without an insert). Right, inhibition of endogenous IGFBP-1 mRNA in HepG2 cells pretreated with anti-*HMGA1* siRNA or a nontargeting control siRNA. WBs of *HMGA1* in each condition are shown in the autoradiograms. β -actin, control of protein loading. * P < 0.05 versus control (black bar); ** P < 0.05 versus siRNA-untreated (control) cells. (b) ChIP of the *IGFBP-1* promoter gene (and a non-AT-rich sequence in the *IGFBP-1* locus, right side) in HepG2 cells, either untreated or pretreated with *HMGA1* siRNA, using an anti-*HMGA1* specific antibody (Ab). Representative assays are shown, together with qRT-PCR of ChIP-ed samples. * P < 0.05 versus control (slashed bar). (c,d) Insulin-mediated IGFBP-1 suppression in HepG2 cells untreated or pretreated with either *HMGA1*-siRNA or distamycin A (100–150 μ M). Conditioned medium samples and cell nuclear extracts were collected after 12 h insulin treatment and assayed by WB for IGFBP-1 and *HMGA1*, respectively. Densitometer scanning of IGFBP-1 signals are shown in bar graphs. Results are expressed as percentages of the IGFBP1 production in the presence of nontargeting siRNA (control) or vehicle alone. * P < 0.05 versus untreated cells. (e,f) Representative ChIPs of the *IGFBP-1* promoter gene with anti-*HMGA1* antibody (Ab) and qRT-PCR of ChIP-ed samples under the same conditions as in (c,d). * P < 0.05 versus untreated cells (slashed bars).

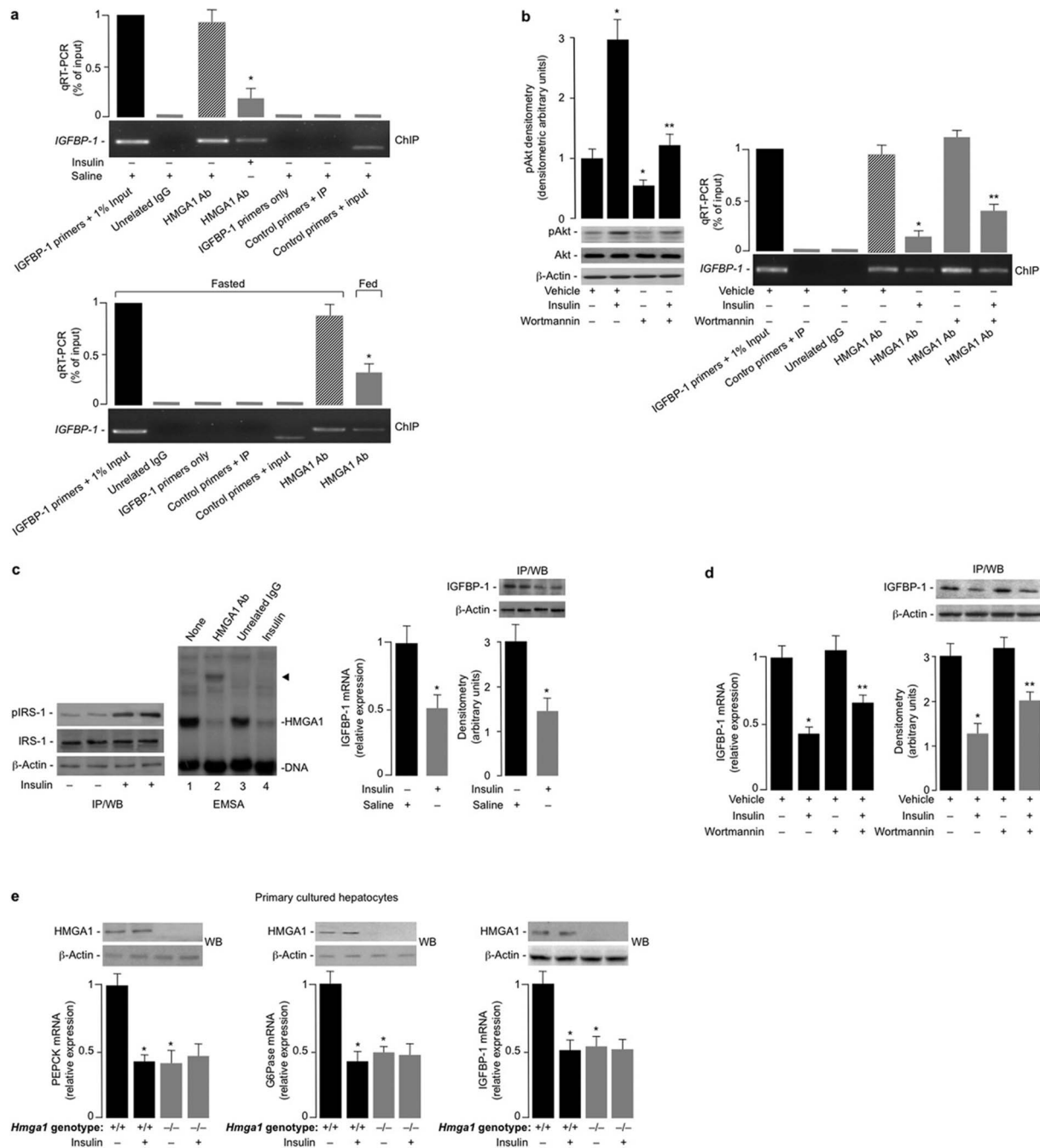


Figure 2 | HMGAI DNA-binding activity and function is regulated by insulin. (a) Representative ChIPs of *IGFBP-1* with anti-HMGAI antibody (Ab). Top, fasted mice were intraperitoneally injected with insulin (1 U/kg bw, $n = 12$) 4 h prior to sacrifice. Bottom, ChIP was performed in mice under fasted ($n = 10$) and fed ($n = 12$) states. qRT-PCR of ChIP-ed samples is shown in each condition. $*P < 0.05$ versus controls (slashed bars) (b) Akt activity and HMGAI-DNA binding. Groups of fasted mice ($n = 6$ each) were injected i.v. with or without wortmannin, followed by insulin injection. Left, WB of total (Akt) and phosphorylated (pAkt) Akt in liver lysates from untreated and treated mice. Densitometric quantifications of three independent experiments from six animals per group are shown. β -actin, control. $*P < 0.05$ versus control vehicle alone; $**P < 0.05$ versus vehicle plus insulin. Right, representative ChIP of *IGFBP-1* with anti-HMGAI antibody (Ab) and qRT-PCR of ChIP-ed samples in liver from treated and untreated mice. $*P < 0.05$ versus untreated mice (slashed bar). $**P < 0.05$ versus insulin alone. (c) Left, IP/WB of phosphorylated (pIRS-1) and total IRS-1 in liver from mice injected or not with insulin, and HMGAI-DNA binding (EMSA) of nuclear extracts from untreated (lanes 1, 2 and 3) and insulin-treated (lane 4) mice. Supershifting of the HMGAI-DNA complex (arrowhead) is shown by using anti-HMGAI antibody (Ab). Control (unrelated rabbit serum IgG) antibody did not alter the mobility of the complex. Right, liver *IGFBP-1* mRNA abundance was analysed by qRT-PCR in mice 4 h after intraperitoneal injection of insulin or saline. A representative *IGFBP-1* immunoblot (IP/WB) of whole-cell liver extracts is shown. β -actin, control. Densitometry of four to six independent blots is provided. $*P < 0.05$ versus untreated (saline) mice. (d) Liver *IGFBP-1* mRNA and protein (IP/WB) levels were measured as in (c), in insulin-injected mice, in the absence or presence of wortmannin. Densitometry of six independent blots is shown. $*P < 0.05$ versus untreated (vehicle) mice. $**P < 0.05$ versus insulin plus vehicle. (e) Insulin-mediated gene suppression in primary cultured hepatocytes from wild-type (+/+) and *Hmgai1*-null (-/-) mice. The mRNA levels of PEPCK, G6Pase and *IGFBP-1* were measured by qRT-PCR in primary cultured cells untreated or treated with 10 nM insulin for 12 h. Data are shown as the means \pm s.e.m. of five independent experiments. $*P < 0.05$ versus untreated (+/+) cells. Protein expression of HMGAI from primary hepatocytes is shown in WBs. β -actin, control.

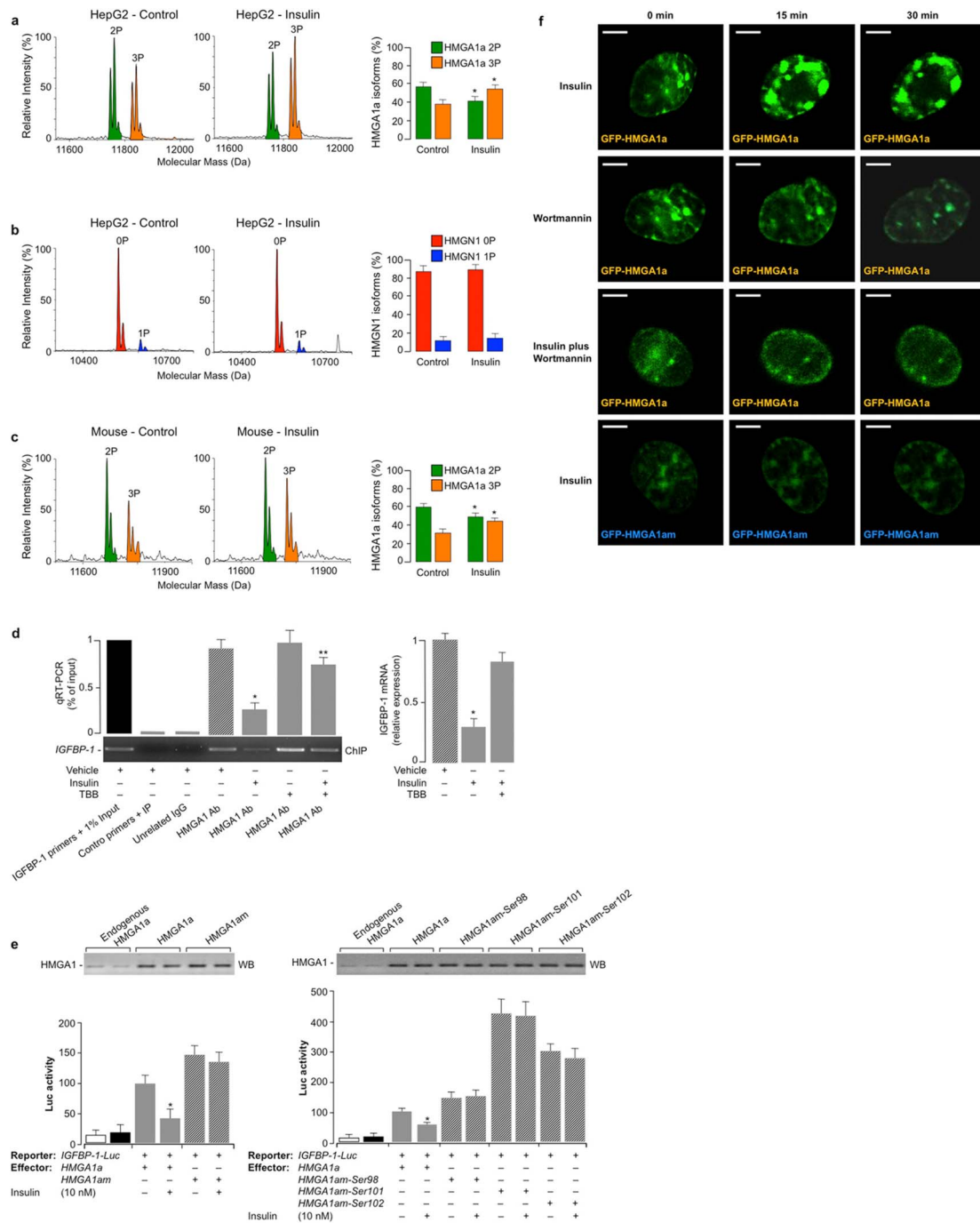


Figure 3 | Insulin-induced HMGA1 phosphorylation and its intranuclear distribution in living cells. (a–b) HMG proteins (A1a and N1) from untreated (control) and 30 min insulin-treated HepG2 cells were purified by RP-HPLC and analysed by mass spectra. (c) Mass spectra of liver HMGA1a protein from saline (control) and insulin-injected wild-type mice. Livers from five animals were pooled for each determination. Abundances of di- (2P, in green) and tri-phosphorylated (3P, in orange) HMGA1a isoforms, together with the unmodified (0P, in red) and mono-phosphorylated (1P, in blue) HMGN1 isoforms are shown as bar graphs. (d) Representative ChIP of *IGFBP-1* with anti-HMGA1 antibody (Ab) and qRT-PCR of ChIP-ed samples and endogenous *IGFBP-1* mRNA (right side) in HepG2 cells untreated or treated with insulin, in the presence or absence of the protein kinase CK2 inhibitor TBB. * $P < 0.05$ versus untreated cells (slashed bars), in each assay; ** $P < 0.05$ versus insulin alone. (e) Expression and function of HMGA1a (wild-type) and its triple (HMGA1am) and single (HMGA1am-Ser) mutants as indicated on the top of WB, in HEK-293 cells, barely expressing endogenous HMGA1a. Cells were cotransfected with *IGFBP-1-Luc* reporter vector and equal amounts of either HMGA1a wild-type (grey bars) or HMGA1a mutants (slashed bars) expression plasmid. At 48 h after transfection, cells were incubated in the absence (–) or presence (+) of insulin and cell lysates were prepared 4 h later. Cell lysates were divided into two aliquots; one of these aliquots was used for Luc activity, and the other was used for WB analysis as a control of HMGA1 protein expression. Luc activity in each condition is expressed as a percentage of the reporter activity obtained in transfections with the wild-type (HMGA1a) effector vector, in the absence of insulin. White bars, mock (no DNA); black bars, pcDNA3 vector without an insert. Data represent the means \pm s.e.m. for three separate experiments. Representative WBs of endogenous and overexpressed HMGA1a and HMGA1a mutant proteins are shown. * $P < 0.05$ versus insulin-untreated control (HMGA1a) cells. (f) Time lapse imaging of intranuclear distribution of GFP-HMGA1a in living HepG2 cells, after treatment (0, 15 and 30 min) with either insulin (10 nM) or wortmannin (100 nM), alone, or a combination of both. Intranuclear distribution of the triple mutant GFP-HMGA1am, in the presence of insulin, is shown. Pictures are optical sections made with a confocal laser scanning microscope. Bars correspond to 2 μ m.



known to be critical for HMGA1-DNA contact^{10,20}. Constitutive and inducible phosphorylation at this level involves the serine residues Ser98, Ser101 and Ser102 and it has been demonstrated to be dependent on PI-3K via a casein kinase 2 (CK2)-like specificity^{10,21}. Whether detachment of HMGA1 from DNA is due to a direct or indirect mechanism, our data demonstrate that phosphorylation of the C-terminal tail is linked to this event. This view was supported by experiments performed with the highly specific CK2 inhibitor 4,5,6,7-tetrabromo-1H-benzimidazole (TBB), showing that both detachment of HMGA1 from DNA and inhibition of endogenous IGFBP-1 mRNA by insulin were prevented by inhibiting CK2 kinase activity (Fig. 3d), while the cyclin-dependent kinase inhibitor alsterpaullone and the protein kinase C (PKC) inhibitor Gö6976 had no effects on these functions (Supplementary Fig. S3). The role of the multiple serine residues Ser98-Ser101-Ser102 on insulin action has been confirmed in functional studies in which simultaneous substitution of all three residues with nonphosphorylatable alanines prevented insulin-inhibition of IGFBP-1-Luc reporter activity in transfected HEK-293 cells (Fig. 3e). When examined individually, the single substitution mutants (Ser98 → Ala, Ser101 → Ala, Ser102 → Ala) also repressed insulin inactivation of IGFBP-1-Luc (Fig. 3e), indicating that the phosphorylation of Ser98, Ser101 and Ser102 are each indispensable for insulin activity. Differences in the magnitude of Luc activity in HEK-293 cells transfected with single-substitution mutants suggest that transactivation by HMGA1 is dependent on both the number and position of phosphate groups on the HMGA1 C-terminal tail. This is consistent with our previous finding which showed that phosphorylation of the multiple serine residues at the HMGA C-terminus is not a random event, as the phosphorylation of a serine residue can influence phosphorylation at an adjacent site⁹. Also, these results confirm the current view on the role of the acidic C-terminal tail phosphorylation in the negative modulation of HMGA DNA-binding properties and thus in their transactivation ability^{9,20}. No hyperphosphorylation of HMGA1a, besides that occurring at the C-terminal tail, was detected after insulin treatment. On the contrary, a slight decrease in the phosphorylation status of some peptides (aa 1–6, 7–14, 18–22, 30–54, and 71–73) was detected (Supplementary Fig. S4 and Supplementary Table S1). Some of them contain serine/threonine residues near the HMGA1 DNA-binding domains, supporting the notion that decreased HMGA1a DNA-binding affinity after insulin treatment is dependent on phosphorylation of the acidic C-terminal tail of HMGA1 (Supplementary Data).

The effect of insulin-induced phosphorylation on nuclear distribution of HMGA1 was then investigated in living cells. Analysis with the green fluorescent protein (GFP)-tagged HMGA1a (GFP-HMGA1a) revealed that, in serum-starved HepG2 cells, the GFP-HMGA1a fusion protein was preferentially located within the transcriptionally active euchromatin in the nuclear interior (Fig. 3f). After 15 min of insulin treatment there was a marked redistribution of GFP-HMGA1a from this site to the repressed inactive heterochromatin, in a circumferential distribution within the nucleus, becoming more evident at 30 min (Fig. 3f). Pretreatment with the PI-3K inhibitor wortmannin totally reversed insulin-induced heterochromatin foci formation and resulted in a more diffuse and homogenous distribution of GFP-HMGA1a throughout the entire nucleus similarly to that in starved control cells (Fig. 3f). To verify the specificity of insulin-induced phosphorylation on nuclear localization of HMGA1, we examined the nuclear distribution of the mutant GFP-HMGA1am (mimicking dephosphorylation), in which the three active serine phosphorylation sites at the C-terminus were mutated to nonphosphorylatable alanines. As shown in Fig. 3f, insulin elicited no effect on this mutant, confirming that phosphorylation at these sites was essential for regulating HMGA1 activity by insulin. Mobility analysis by photoactivation confirmed that, in serum-starved HepG2 cells, the photoactivable GFP protein

(PAGFP)-tagged HMGA1a (PAGFP-HMGA1a) was preferentially located in the nuclear interior (Supplementary Video S1). Once again, insulin treatment caused a marked redistribution of PAGFP-HMGA1a from the nuclear interior site to the peripheral region of the nucleus, and this effect was reversed by wortmannin and was prevented in cells expressing the mutant PAGFP-HMGA1am protein (Supplementary Video S1). Thus, these results indicate that insulin is mechanistically involved in the dynamic interaction of HMGA1 with DNA/chromatin *in vivo*.

Insulin-induced INSR downregulation is mediated by HMGA1 phosphorylation.

Downregulation of INSR mRNA by insulin has been demonstrated *in vitro* with several cell types²². Indeed, we previously showed that mRNA for the INSR was upregulated in insulin-starved mammalian cells^{23,24}, in which this rise was paralleled closely by a rise in a small nuclear protein (later found to be HMGA1)^{11,24}. In the present work, to probe further the relevance of HMGA1 for the INSR signaling system, we carried out studies in cultured HepG2 cells as well as in mice, demonstrating that phosphorylation of HMGA1 is an essential and necessary prerequisite for the insulin-induced INSR downregulation either *in vitro* or *in vivo*. As shown in Fig. 4a, HepG2 cells upregulate the INSR mRNA in the absence of serum, conditions that induce the dephosphorylation and activation of HMGA1; conversely, insulin was able to reverse this effect. Inhibition of INSR expression by insulin was abolished in HepG2 cells almost completely depleted of HMGA1 (Fig. 4a). Consistently with our model, inhibition of INSR mRNA by insulin in HepG2 cells paralleled closely the decrease in HMGA1 occupancy at the endogenous *INSR* locus (Fig. 4a). In concert with these observations, INSR mRNA abundance was increased in liver from 12 h-fasted normal mice (Fig. 4b,c) and was reduced in mice with augmented insulin levels as obtained following either systemic administration of insulin (Fig. 4b), or after meal ingestion (Fig. 4c). As with *IGFBP-1* gene, binding of endogenous HMGA1 to the *INSR* gene locus was increased in liver from mice under physiological fasting conditions, when the insulin signaling cascade is attenuated (Fig. 4b,c), and promptly decreased following insulin injection (Fig. 4b) or after refeeding (Fig. 4c), when insulin signaling is reactivated, indicating that HMGA1 acts as a molecular switch for activating or deactivating INSR expression during the fasting and refeeding periods, respectively. In line with this, insulin failed to suppress INSR mRNA in the absence of HMGA1, as observed in primary cultured hepatocytes from HMGA1-null mice (Fig. 4d), and no changes on INSR mRNA levels were observed in insulin-treated HEK-293 cells overexpressing single (HMGA1am-Ser) or triple (HMGA1am) HMGA1 protein mutants (Fig. 4d).

Thus, these data collectively underscore the importance of the architectural factor HMGA1 in the context of the INSR signaling pathway, and establish HMGA1 as a novel key player in the nutritionally and insulin-regulated transcription of genes involved in glucose metabolism.

Discussion

The intracellular signaling pathways by which changes in gene expression are triggered by insulin are only partly identified and more investigations are needed to decipher the molecular mechanisms of this transcriptional regulation. The initial interaction of insulin with target cells is via its receptor located in the plasma membrane^{25–27}. Upon binding of insulin, the INSR undergoes autophosphorylation which enables the receptor to have a kinase activity and phosphorylates various cytoplasmic INSR substrates (IRs). From this point, signaling proceeds via a variety of signaling pathways (i.e. PI-3K signaling pathway, Ras and MAP kinase cascade) that are responsible for the metabolic, growth-promoting and mitogenic effects of insulin. Based on these considerations, it is possible,

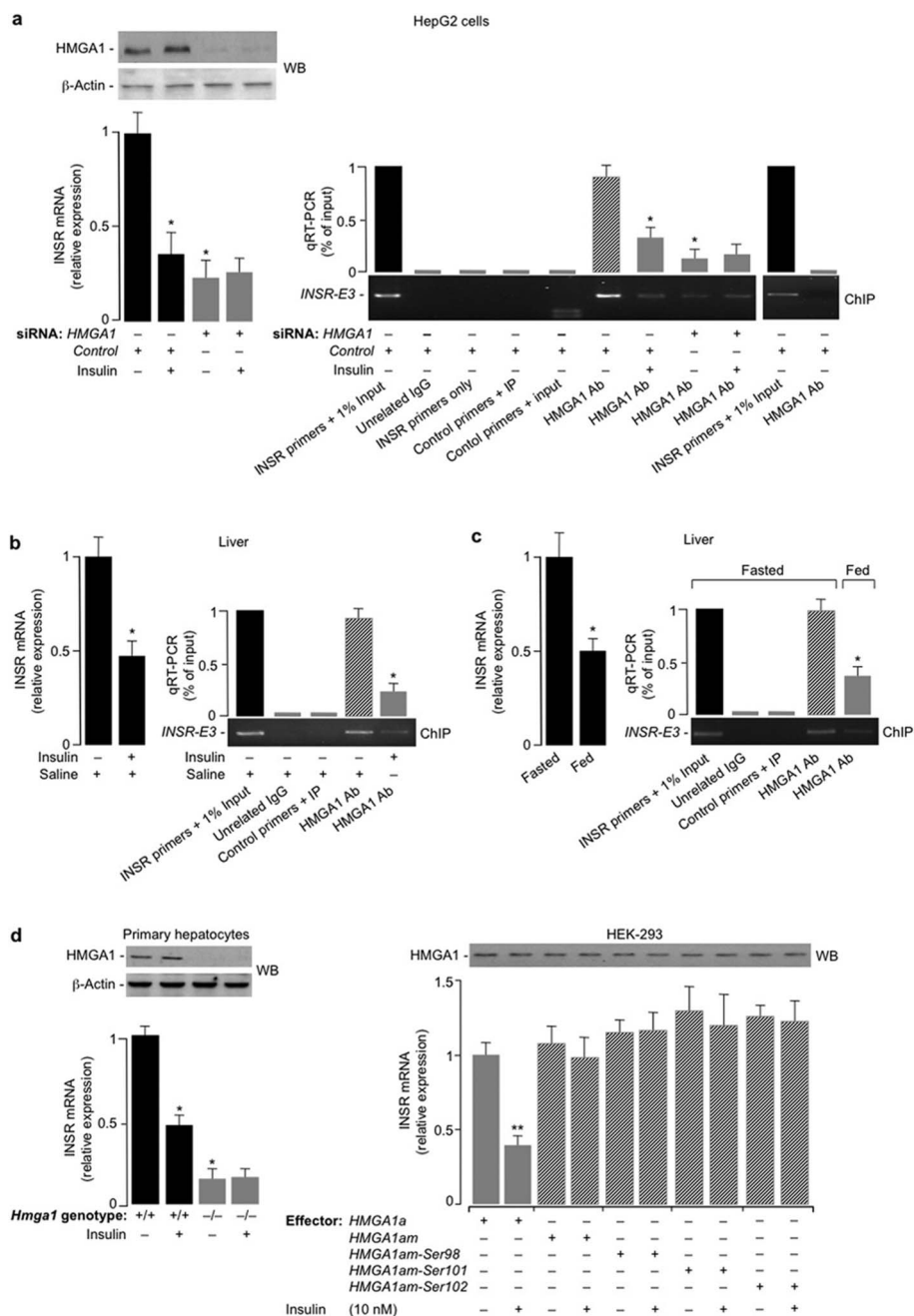


Figure 4 | Insulin-induced INSR downregulation is mediated by HMGA1 phosphorylation. (a) Left, serum-starved HepG2 cells, either untreated or pretreated with HMGA1-siRNA, were incubated without or with insulin (10 nM) for 12 h. Total RNA was extracted, and mRNA for the INSR was quantitated by qRT-PCR. *RPS9* mRNA was used to normalize. Data represent mean \pm s.e.m. of three independent experiments; $*P < 0.05$ versus insulin/siRNA-untreated cells. Representative WB of HMGA1 protein expression is shown in the autoradiogram. β -actin, control of protein loading. Right, ChIP shows that binding of HMGA1 to the *INSR* gene promoter (*INSR-E3*) was decreased in serum-starved HepG2 cells treated with insulin. A representative assay out of three independent experiments is shown. Right side, ChIP with a non-AT-rich sequence in the *INSR* locus. qRT-PCR of ChIP-ed samples is shown in each condition. $*P < 0.05$ versus insulin/siRNA-untreated sample (slashed bar). (b) 12 h-fasted wild-type mice were intraperitoneally injected with either insulin (1 U/kg bw) or saline and sacrificed 2–4 h later. Left, total RNA was isolated from liver, INSR mRNA was measured by qRT-PCR and normalized to *RPS9* mRNA abundance. Results are the mean values \pm s.e.m. from six animals per group. $*P < 0.05$ versus untreated (saline) control mice. Right, occupancy of the *INSR-E3* gene promoter by HMGA1 as measured by ChIP with anti-HMGA1 specific antibody (Ab) in liver from mice after treatment with saline or insulin. A representative assay is presented. qRT-PCR of ChIP-ed samples is shown in each condition. $*P < 0.05$ versus insulin-untreated sample (slashed bar). (c) Left, liver INSR mRNA was assayed as in (b) in 12 h-fasted mice ($n = 6$), and mice refed for 4 h ($n = 5$) with a high carbohydrate diet after a 12-h fast. $*P < 0.05$ versus fasted mice. Right, phosphorylation change affecting the interaction of HMGA1 with the *INSR-E3* gene promoter during the fast/fed transition is shown by ChIP *in vivo*, on whole liver. qRT-PCR of ChIP-ed samples is shown in each condition. $*P < 0.05$ versus fasted sample (slashed bar). (d) Insulin-mediated INSR mRNA downregulation in primary cultured hepatocytes from wild-type (+/+) and *Hmga1*-null (-/-) mice (left), and in HEK-293 cells overexpressing either wild-type (HMGA1a), triple (HMGA1am) or single (HMGA1am-Ser) HMGA1a mutants (right). INSR mRNA levels were measured by qRT-PCR in cells untreated or treated with 10 nM insulin for 12 h. In each condition, data are shown as the means \pm s.e.m. of five independent experiments. $*P < 0.05$ versus untreated (+/+) cells; $**P < 0.05$ versus insulin untreated (HMGA1a) control. Protein expression of HMGA1 in primary hepatocytes and HEK-293 cells is shown by WB. β -actin, control.



therefore, that insulin-regulated transcription of genes involved in glucose metabolism may result, at least in part, from posttranslational modifications, including phosphorylation, that can affect access of transcription factors to DNA, thereby silencing and/or unsilencing gene expression. This possibility, that would account for the pleiotropic effects of this hormone, is greatly supported by our current findings here, indicating that, by inducing HMGA1 protein phosphorylation, insulin is directly and mechanistically involved in the dynamic interaction of HMGA1 with DNA/chromatin *in vivo*, thus in the control of gene activity.

Here we provide evidence that HMGA1 plays an essential role in the transcriptional regulation of a variety of insulin-target genes such as *IGFBP-1* and *INSR* genes, as well as the gluconeogenic genes *PEPCK* and *G6Pase*. IGFBP-1 is distinctive among the IGFBPs, as its plasma concentrations show marked diurnal variations due to hormonal and metabolic changes. IGFBP-1 is thought to be the primary IGF binding protein involved in the acute regulation of serum glucose levels²⁸. Fasting hyperglycemia with impaired glucose tolerance and insulin resistance has been demonstrated in rats after the injection of IGFBP-1²⁸, as well as in transgenic mice overexpressing IGFBP-1²⁹, in the presence of reduced concentrations of circulating free IGF-I. Given that IGFBP-1 serum levels were considerably decreased in *Hmga1*-knockout mice¹⁷ and IGFBP-1 mRNA expression was also reduced in HepG2 cells by siRNA targeting *HMGA1*, it appears reasonable to interpret that insulin-induced functional inactivation of HMGA1, by adversely affecting the production of IGFBP-1 protein species, could serve as a mechanism to increase the insulin-like effects of endogenous IGF-I, thereby contributing to the maintenance of postprandial glucose homeostasis. The contribution of HMGA1 to the postprandial regulation of glucose homeostasis is supported by our observations in primary cultured hepatocytes, indicating that repression of the gluconeogenic genes *PEPCK* and *G6Pase* by insulin requires HMGA1. Consistent with these observations, we previously found that the counter-regulatory hormone glucagon, which acts in opposition to insulin to maintain a euglycemic (normal) state, upregulates HMGA1 protein production *in vivo* in whole mice³⁰. Based on our findings here, it is likely that upregulation of HMGA1 during fasting (when glucagon peaks) may also contribute to the maintenance of fasting euglycemia. In addition, the hypothesis that HMGA1, by increasing fasting serum IGFBP-1, may contribute to restrict glucose uptake by peripheral tissues, thereby sparing glucose for the brain and other glucose-dependent tissues, is also supported.

The molecular mechanisms regulating *INSR* gene expression have been elucidated and evidence has been provided showing that HMGA1 is required for proper transcription of the *INSR* gene in mammals^{11,12}. HMGA1 acts on the *INSR* gene promoter as an element necessary for the formation of a transcriptionally active multi-protein-DNA complex involving, in addition to the HMGA1 protein, the ubiquitously expressed transcription factor Sp1 and the CCAAT-enhancer binding protein beta (C/EBP β)¹². By potentiating the recruitment and binding of Sp1 and C/EBP β to the *INSR* promoter, HMGA1 greatly enhances the transcriptional activities of these factors in the context of the *INSR* gene¹². As demonstrated in this study, consistently with the observation that functional repression of endogenous HMGA1 is strictly dependent on insulin-induced HMGA1 phosphorylation, *INSR* gene expression was considerably reduced *in vitro*, in insulin-treated HepG2 cells, and *in vivo*, in liver from normal mice with augmented insulin levels as obtained following insulin injection or after a meal.

The possibility that intracellular regulatory molecules that are employed by the *INSR* signaling system could be involved in post-translational modifications of HMGA1, including protein phosphorylation, has been postulated before, on the basis of experimental observations *in vitro*, involving the novel PKC ϵ isotype that has recently been shown to be linked to the insulin signaling pathway³¹.

The literature, however, does not provide explanation on how these modifications occur and function in concert. Herein, we provide data that help explain these issues and add new insights into the physiological mechanistic details governing *INSR* signal transduction. In view of our results on the HMGA1 post-translational modifications, it appears likely that variations in the biological activity of HMGA1 can be specifically produced in response to hormonal and nutritional changes through mechanisms of phosphorylation/dephosphorylation of the HMGA1 protein, leading to gene repression or activation, respectively. These findings may not only play a role under physiological circumstances related to acute metabolic and hormonal responses (e.g., after a meal, or when insulin decreases during fasting), but may also contribute to pathophysiological pathways in a range of blood sugar-related disorders, in which insulin levels are chronically elevated. In this regard, data have been published indicating that type 2 diabetes mellitus may occur in humans and mice carrying *HMGA1* gene defects, in which abnormalities of both the insulin-receptor signaling system and the IGF-I-IGFBP-1 system were identified^{17,32,33}.

The FOXO (forkhead) family of transcription factors are critical regulators of insulin action, and cytoplasm retention of FOXOs via phosphorylation is suggested to be a mechanism of insulin-mediated *IGFBP-1* and *INSR* gene repression³⁴⁻³⁶. The possibility that an interplay among HMGA1 and FOXO can be a component of this regulation constitutes an interesting point that will deserve further consideration. Compared to previous investigations, for the first time in the present study, we report the identification of HMGA1 as a novel downstream nuclear target of the *INSR* signaling pathway, which may play a major role in insulin-dependent gene expression and regulation in mammals. This, in our opinion, is interesting from both biological and mechanistic points of view and might be useful in understanding the molecular basis of clinical phenotypes in certain conditions where insulin action becomes compromised (*i.e.* diabetes mellitus, obesity and other insulin-resistant syndromes). Understanding these mechanisms should augment our capacity to identify novel therapeutic targets for the prevention and treatment of these diseases.

Methods

Plasmid construction and transfections, nuclear distribution and photoactivation analysis and qRT-PCR. Primers used for plasmid construction of human *IGFBP-1* promoter-containing vector: 5'-TAGCCCTGAGCTCTGCTAG-3' (containing Sac I restriction site) and 5'-ACAGGGGCCGAGCTTCTGG-3' (containing Hind III restriction site). PCR products were subcloned into pGL3-basic vector (Promega) at the indicated restriction sites. Recombinant Luc reporter construct in the presence or absence of effector vector for HMGA1 (HMGA1a isoform protein)¹², were transiently transfected into HepG2 cells, using LipofectAMINE 2000 reagent (Invitrogen), and Luc activity was assayed 48 h later in a luminometer (Turner Biosystems), using the dual-luciferase reporter assay system (Promega). siRNA targeted to human *HMGA1*³⁷, plus nonspecific siRNA controls with a similar GC content were obtained from Dharmacon. 100–200 pmol siRNA duplex was transfected into cells at 40% to 50% confluency. After 1 h knockdown for 72 h, the cells were trypsinized, pooled, and resuspended for a second transfection using the same targeting siRNA. After an additional 72 h, cells were prepared for analysis. Renilla control vector served as an internal control of transfection efficiency, together with measurements of protein expression levels. Site-directed mutagenesis of the serine phosphorylation sites of HMGA1a was carried out by using the site-directed mutagenesis kit (Stratagene) with the following pair of PCR primers (5'-AAGAGGAGGGCATCGCGCAGGAGGCCGCGGAGGAGGAGCAG-3' and 5'-CTGCTCCTCTCCGCGGCCTCTGCGCGATGCCCTCTCTT-3', sites for mutagenesis are underlined) to yield single and triple HMGA1a mutants.

To produce GFP-HMGA1a and PAGFP-HMGA1a expression plasmids, the human HMGA1a ORF (NCBI Ref. Seq. NM_145899.2) was cloned into BamHI/XbaI sites of pGFP (Clontech) or PAGFP expression vector (a kind gift from Dr. Faretta, European Institute of Oncology, Milan, Italy), respectively. HepG2 cells were transfected with either wild-type or mutant GFP-HMGA1a or PAGFP-HMGA1a effector plasmid, spotted the day after on glass bottom poly-D-lysine coated plates (Met-Tek Corporation), and used for time-lapse imaging or photoactivation studies, respectively. For time-lapse studies, images were collected on a Leica TCS-SP2 confocal microscope (Leica Microsystems) with a 63x Apo PLA oil immersion objective (NA 1.4) and 60- μ m aperture. GFP-expressing cells were visualized by excitation with an argon laser at 488 nm and photomultiplier tube voltage of 420 mV. For photoactivation studies, transfected cells were first identified by scanning for low levels of



expression of PAGFP-SmB using a 405 pulse at 5% laser power, then confocal analysis was performed on selected cells following cell activation by a 200 msec 406 nm pulse at 100% laser power focused to a spot of approximately 1.5 μm diameter. A series of single Z-sections of each cell was recorded over 1 min after photoactivation using a FITC filter.

For qRT-PCR, total cellular RNA was extracted from cells and tissue using the RNAqueous-4PCR kit and subjected to DNase treatment (Ambion). RNA levels were normalized against 18S ribosomal RNA in each sample, and cDNAs were synthesized from 2 μg of total RNA using the RETROscript first strand synthesis kit (Ambion). Primers for mouse *IGFBP-1* (NM_008341) (5'-CCTAACTGTTGTTCTTGGC-3'; 5'-AGAAATCTCGGGCACGAA-3'), human *IGFBP-1* (NM_000596.2) (5'-CATTCATCCTTTGGGAC-3'; 5'-ATTCTGTGTGAGTTTGGCAG-3'), human *INSR* (NM_000208.2) (5'-TTTGGGAAATCACCAGCTTGGCAGAA-3'; 5'-AAAGCTGGGGTGCAGGTC GTCTTG-3'), mouse *INSR* (NM_010568) (5'-TATGACGACTGCCAGTGA-3'; 5'-ATCTGGAAAGTGTGAGTGTGG-3'), mouse *PEPCK1* (NM_011044) (5'-GTGTCATCCGCAAGCTGAAGA-3'; 5'-CTTTCGATCCTGGCCACATCT-3'), mouse *G6Pase* (NM_008061) (5'-CTGCCAGGGAGAACTCAGCAA-3'; 5'-GAGGACCAAGGAAGCCACAAT-3') were designed according to sequences from the GenBank database. A real-time thermocycler (Eppendorf Mastercycler ep realplex ES) was used to perform quantitative PCR. In a 20- μl final volume, 0.5 μl of the cDNA solution was mixed with SYBR Green RealMasterMix (Eppendorf), and 0.3 μM each of sense and antisense primers. The mixture was used as a template for the amplification by the following protocol: a denaturing step at 95°C for 2 min, then an amplification and quantification program repeated for 45 cycles of 95°C for 15 s, 55°C for 25 s, and 68°C for 25 s, followed by the melting curve step. SYBR Green fluorescence was measured, and relative quantification was made against *RPS9* cDNA used as an internal standard. All PCR reactions were done in triplicates.

Animals. All animal work was conducted according to institutional guidelines for the care of laboratory animals. For the wortmannin studies, wild-type mice were given single bolus injections of wortmannin (1 mg/kg bw) or vehicle (0.5% DMSO) alone via the tail vein, followed 1 h later by an intraperitoneal injection of insulin (1 U/kg bw). The liver was harvested 4 h after insulin injection to determine Akt phosphorylation and HMGA1-DNA binding, in addition to IGFBP-1 expression. *INSR* mRNA and ChIP of the *INSR* promoter gene were analysed in liver from 12 h-fasted mice after intraperitoneal injection of insulin (1 U/kg bw) or saline and after 4 h refeeding. Mouse hepatocytes were isolated as described elsewhere³⁸. Primary hepatocytes were cultured on matrigel-coated six-well plates in Williams E media (Sigma) supplemented with 10% FBS. $1.5\text{--}2.5 \times 10^6$ cells were allowed to adhere for 2–3 h and were then incubated in serum-free DMEM containing 0.1% BSA. Cells were maintained in this medium overnight (12 h) and then utilized in subsequent experiments.

ChIP. ChIP assay was performed in cultured HepG2 cells, either untreated or pretreated with *HMGA1* siRNA as described previously^{17,39}. For *in vivo* ChIP, at the end of the indicated treatments, mice were killed by cervical dislocation, the liver was rapidly removed, prewarmed with PBS, and treated with collagenase (0.2%) for 15 min. The liver was then diced, forced through a 60 μm stainless steel sieve, the hepatocytes were collected directly into DMEM containing 1% formaldehyde, and the formaldehyde-fixed DNA-protein complexes were immunoprecipitated with anti-HMGA1 antibody^{17,39}. Sequence-specific primers for *IGFBP-1* and *INSR* gene promoters used for PCR amplification of ChIP DNA (30 cycles), using PCR ready-to-go beads (Amersham Pharmacia Biotech): human *IGFBP-1* (NT_007819) for 5'-CAGAAAGAGAAGCAATTCG-3', rev 5'-TACCAGCCAGACGGCAGCAA-3'; mouse *IGFBP-1* (NT_039515) for 5'-CCTGGGGAGGGAGAAACAAC-3', rev 5'-GCAGTGTCAATGCTCGCTGG-3'; human *INSR* (NT_11255.14) for 5'-AGATCTGGCCATTGCAC-3', rev 5'-ATGCCAGTCTGGGAGGTA-3'; mouse *INSR* (NC_000074.5) for 5'-TTGTTGGGCGCTACTAGC-3', rev 5'-AAACACAAGTAACACCGAGG-3'. Primers for ChIP with non-AT-rich sequences: human *IGFBP-1* locus [for 5'-AGTAGAGATGGGGTTTGGC-3' (-2266/-2247 from the ATG), rev 5'-GATAGCAATGCCTTCTGTG-3' (-2022/-2003 from the ATG)]; human *INSR* locus [for 5'-TCCCTGCAAGCTTTCCCTC-3' (-586/-567 from the ATG), rev 5'-TACTGAGCGGAGGCCCTTGGCGGT-3' (-226/-204 from the ATG)]. PCR products were electrophoretically resolved on 1.5% agarose gel and visualized by ethidium bromide staining. Primers for qRT-PCR ChIP-ed samples are available upon request.

IP/WB. These assays were performed to analyse IGFBP-1 protein expression in HepG2 cells and in whole-cell liver extracts (1 mg) from treated and untreated mice, using a polyclonal specific antibody raised against IGFBP-1 (Santa Cruz Biotechnology). For the measurement of IRS-1 and Akt phosphorylation, protein extracts (1 mg) from mice were prepared and analysed as previously described¹⁷. IRS-1 tyrosine phosphorylation was assayed after IP of total lysates with antibody to IRS-1, followed by immunoblotting with anti-phosphotyrosine antibody. WBs for Akt were performed using antibody that recognizes only phosphorylated Ser473 form of Akt and antibody that recognizes total (nonphosphorylated and phosphorylated) Akt in liver. The antibodies used for these studies were: anti-phosphotyrosine, anti-IRS-1, anti-Akt, anti-pAkt (Upstate Biotechnology). A polyclonal-specific antibody against HMGA1¹¹ was used to analyse HMGA1 protein expression in HepG2 cells.

EMSA. Nuclear extracts were prepared from liver of wild-type animals, injected or not with insulin, and binding of HMGA1 to DNA was assessed using a ³²P-labeled oligonucleotide duplex corresponding to the human *INSR E3* promoter element^{11,24}. In supershift assays nuclear protein was preincubated with 1 μg polyclonal antibody to HMGA1 before addition of the probe.

HMGA protein extraction, HPLC, LC-MS and LC-MS/MS analyses. HMG proteins from HepG2 cells were selectively extracted by treating cells with 5% perchloric acid (PCA), precipitated by acetone-HCl, resuspended in water and their presence confirmed by SDS-PAGE⁹. By employing these conditions, endogenous HMG proteins were also extracted from the whole liver of wild-type mice intraperitoneally injected with insulin (1 U/kg bw) or saline after 12 h fasting. Reverse-phase HPLC chromatography and LC-MS analyses were carried out using a PerkinElmer Life Sciences apparatus (Series 200 LC Pump), using a RP Supelco Discovery BIO Wide Pore C5 column (2.1 \times 250 mm, 5 μm) directly interfaced with an ESI interface to an ion trap HCTultra mass spectrometer (Bruker Daltonics). HPLC separations were carried out using a water/acetonitrile gradient with 0.05% TFA as a modifier. MS parameters were the following: full MS scan: m/z 600–1100; mass range mode: standard/enhanced; ion charge control (ICC) active allowing the storage of a maximum of 200000 ions in a maximum accumulation time of 200 msec. LC-MS were elaborated with Data Analysis software (version 3.4, Bruker Daltonics). Identities of proteins were obtained by comparison of theoretical with experimental molecular masses, taking into consideration combinations of the various post-translational modifications. LC-MS/MS analyses were performed as previously described⁹ and were used to map the phosphorylation increase in HMGA1a sequence. HMGA1a protein was extracted from control and insulin treated HepG2 cells and purified (HPLC). Equal amounts of HMGA1a were then trypsin digested and LC-MS/MS analysed. Among all the peptides found, the phosphorylated counterparts were searched.

Statistics. Statistical significance was evaluated using a 2-tailed Student's *t* test. *P* < 0.05 was considered significant. All bar graph data shown represent mean \pm s.e.m.

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Author contributions

E.C. participated in the analysis and discussion of the data and drafting of the manuscript; M.N. and C.P. performed photoactivation studies; S.I. and F.P. performed western blot, EMSA and transfection studies; E.M., R.S. and G.M. contributed with *in vitro* and *in vivo* studies of post-translation modifications; B.A., K.P. and A.N. participated in ChIP analysis and performed qRT-PCR and cloning studies; D.F. contributed to the data analysis and provided helpful and critical reading of the manuscript; G.B. participated in the analysis and discussion of data from fasting and fed mice; A.B. conceived, coordinated and supervised the study, analysed the data and wrote the paper. All authors discussed the results and commented on the paper.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

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