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

Eusebio Chiefari1, Maria T. Nevolo1, Biagio Arcidiacono1, Elisa Maurizio3, Aurora Nocera1, Stefania Iiritano1, Riccardo Sgarra3, Katiucia Possidente1, Camillo Palmieri1, Francesco Paonessa1, Giuseppe Brunetti4, Guidalberto Manfioletti3, Daniela Foti1 & Antonio Brunetti1,2

1Dipartimento di Scienze della Salute, Università di Catanzaro ‘Magna Graecia’, viale Europa (Località Germaneto), 88100 Catanzaro, Italy, 2Cattedra di Endocrinologia, Università di Catanzaro ‘Magna Graecia’, via Europa (Località Germaneto), 88100 Catanzaro, Italy, 3Dipartimento di Scienze della Vita, Università di Trieste, via Giorgieri 1, 34127 Trieste, Italy, 4Dipartimento di Scienze Biomolecolari e Biotecnologie, Università di Milano, via Celoria 26, 20133 Milano, Italy.

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.

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
examined the effect of wortmannin, a potent and selective inhibitor of PI-3K/Akt, in vivo during fasting. IGFBP-1, a growth factor, may serve to prevent the hypoglycemic effects of IGF-I, thus supporting a physiological role for IGFBP-1 in glucose counterregulation. Insulin plays a major role in the regulation of IGFBP-1, rapidly suppressing its production by the liver at the level of gene transcription. 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 promoter. Consistent with this latter possibility, we previously found that IGFBP-1 expression was considerably reduced in Hmg1a-knockout mice. 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 DNA. 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, did not change its phosphorylation state during insulin treatment (Fig. 2b). 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 HMGA1a 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 examples. 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 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 Hmg1-a null mice. As shown in Fig. 2e, mRNA expression of PEPCK, G6Pase and IGFBP-1 was lower in primary hepatocytes from Hmg1-a 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 HMGA1 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 HMGN1, 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 examples. 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) Human IGFBP-1-Luc reporter vector (2 μg) was transfected into HepG2 cells, plus increasing amounts (0, 0.5 or 1 μg) of HMGA1 effector plasmid. Total plasmid DNA amounts were normalized with empty vector and Luc activity was assayed. Data represent the means ± 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 μM). 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 | HMGA1 DNA-binding activity and function is regulated by insulin. (a) Representative ChIPs of IGFBP-1 with anti-HMGA1 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 HMGA1-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-HMGA1 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 HMGA1-DNA binding (EMSA) of nuclear extracts from untreated (lanes 1, 2 and 3) and insulin-treated (lane 4) mice. Supershifting of the HMGA1-DNA complex (arrowhead) is shown by using anti-HMGA1 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 Hmga1-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 ± s.e.m. of five independent experiments. *P < 0.05 versus untreated (+/+ ) cells. Protein expression of HMGA1 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. 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 ± 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 μm.
known to be critical for HMGA1-DNA contact. 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. 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-tetabromo-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 site. 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. 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 photoactivatable 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.

**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. Upon binding of insulin, the INSR undergoes autophosphorylation which enables the receptor to have a kinase activity and phosphorylates various cytoplasmic INSR substrates (IRSs). 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 ± 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 ± 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. Right side, 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 ± 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 HMGAI protein phosphorylation, insulin is directly and mechanistically involved in the dynamic interaction of HMGAI with DNA/chromatin in vivo, thus in the control of gene activity.

Here we provide evidence that HMGAI 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 IGFBP's, 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 levels. Fasting hyperglycemia with impaired glucose tolerance and insulin resistance has been demonstrated in rats after the injection of IGFBP-1, as well as in transgenic mice overexpressing IGFBP-1, in the presence of reduced concentrations of circulating free IGF-I. Given that IGFBP-1 serum levels were to insulin to maintain a euglycemic (normal) state, upregulates consistent with these observations, we previously found that primary IGF binding protein involved in the acute regulation of insulin receptor signaling and the IGF-I–IGFBP-1 system were identified.

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 repression. The possibility that an interplay among HMGAI 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 HMGAI as a novel downstream nuclear target of the INSR signaling pathway, which may play a major role in insulin-dependent gene repression 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′-TACGCCCTGAGGCTCTGAGTT-3′ (including Sac I restriction site) and 5′-ACAGGGCAGACGGTCTGTTTGTG-3′ (including Hind III restriction site). PCR product (from plasmid containing basic vector Promega) at the indicated restriction sites. Recombinant luc reporter construct in the presence or absence of effector vector for HMGAI (HMGAI isoform protein), 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 HMGAI, 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 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 HMGAIa was carried out by using the site-directed mutagenesis kit (Stratagene) with the following pair of PCR primers (5′-AAGAGACCGGCATTCCGCGGCAAGGACGGAAAGCAGCGGCTG - 5′-CTGCCCTCCTCCCGGCGCTCTGAGTGCCCTCCTCTT-3′, sites for mutagenesis are underlined) to yield single and triple HMGAIa mutants.

To produce GFP-HMGAIa and PAGFP-HMGAIa expression plasmids, the human HMGAIa ORF (NCBI RefSeq_NM_145899.2) was cloned into BamH1/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-HMGAIa or PAGFP-HMGAIa 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 PL A oil immersion objective (NA 1.4) and 60-μm 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 nm pulse at 5% laser power, then confocal analysis was performed on selected cells following cell activation by a 200 mW 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 RNeasyau-PCR kit and subjected to DNase treatment (Ambion). RNA levels were normalized against 18S ribosomal RNA and cDNAs were synthesized from 2 μg of total RNA using the RETROscript first strand synthesis kit (Ambion). Primers for mouse IGFBP-1 (NM_000854.1) (5'-CCTACTGTGTTTCTTGGGC-3'), 5'-GAAGATCTGGGGCCAGAAGA-3'), human IGFBP-1 (NM_000596.2) (5'-CAATCACATCGTTGGGAC-3') and (5'-ATCTCTGCTGAGTTGTCGG-3'), human INS (NM_000208.2) (5'-TTTGGAGAAATCCACGTCAGGAC-3') 5'-AAAGCTGGGTTCAGCTGTGTCCTTG-3'), mouse INS (NM_015068), (5'-TATTGAGCTCTGCAGCTAGTA-3') and (5'-ATCGTTGAACTGATGTTGG-3'), mouse Pepck (NM_010944) (5'-GGTGTTCAGCAGCTTAGA-3') and (5'-CTTGGTACGTCCTGACACT-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 denaturation 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 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 injection to determine Act phosphorylation and HMGA1-DNA binding, in addition to IGFBP-1 expression.

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 ± s.e.m.

1. Bustin, M. & Reeves, R. High-mobility group proteins: architectural components that facilitate chromatin function. Prog. Nucleic Acids Res. 54, 35–100 (1996).
2. Reeves, R. Molecular biology of HMGA proteins: hubs of nuclear function. Gene 277, 63–81 (2001).
3. Thanos, D. & Maniatis, T. Virus induction of human IFN beta gene expression requires the assembly of an enhancerosome. Cell 83, 1091–1100 (1995).
4. Oliote, S. A. et al. The DNA-banding protein HMGA1 enhances progestere receptor binding to its target DNA sequences. Mol. Cell. Biol. 14, 3376–3391 (1994).
5. Falvo, J. V., Thanos, D. & Maniatis, T. Reversal of intrinsic DNA binding in the HMG gene beta enhancer by transcription factors and the architectural protein HMGB (Y). Cell 83, 1101–1111 (1995).
6. Theron, S. & Nissen, M. S. Cell cycle regulation and functions of HMGB-Y (Prog. Cell Cycle Res. 1, 339–349 (1991).
7. Huth, J. R. et al. The structure of an HMGB-Y-DNA complex defines a new architectural minor groove binding motif. Nat. Struct. Biol. 4, 657–667 (1997).
8. Edberg, D. D., Bruce, J. E., Stehn, W. F. & Reeves, R. In vivo post-translational modifications of the High Mobility Group A1a proteins in breast cancer cells of different metastatic potential. Biochemistry 43,11500–11515 (2004).
9. Sgarra, R. et al. Macrosopic differences in HMGA oncoproteins post-translational modifications: C-terminal phosphorylation of HMGA2 affects its DNA binding properties. J. Proteome Res. 8, 2978–2989 (2009).
10. Wang, D.-Z., Ray, F. & Boothby, M. Interleukin 4-inducible phosphorylation of HMGA (Y) is Inhibited by Rapamycin. J. Biol. Chem. 270, 22924–22932 (1995).
11. Brunetti, A., Maniobetti, G., Chiefari, E., Goldfield, I. D. & Fott, T. D. 7-translational modifications of the High Mobility Group A1a proteins in breast cancer cells of different metastatic potential. Biochemistry 43,11500–11515 (2004).
12. Edberg, D. D., Bruce, J. E., Stehn, W. F. & Reeves, R. In vivo post-translational modifications of the High Mobility Group A1a proteins in breast cancer cells of different metastatic potential. Biochemistry 43,11500–11515 (2004).
phosphorylation at either serine 28 or 10 by the MSK1 multi-protein complex. Nucleic Acids Res 38, 3196–3208 (2010).
20. Piekielko, A. et al. Distinct organization of DNA complexes of various HMG1/Y family proteins and their modulation upon mitotic phosphorylation. J Biol Chem 276, 1984–1992 (2001).
21. Sgarra, R. et al. Nuclear phosphoproteins HMGA and their relationship with chromatin structure and cancer. FEBS Lett 574, 1–8 (2004).
22. Pender, C. et al. Muscle insulin receptor concentrations in obese patients post bariatric surgery: relationship to hyperinsulinemia. Int. J. Obes. Relat. Metab. Disord. 28, 363–369 (2004).
23. Brunetti, A., Maddux, B. A., Wong, K. Y. & Goldfine, I. D. Muscle cell differentiation is associated with increased insulin receptor biosynthesis and messenger RNA levels. J. Clin. Invest. 83, 192–198 (1989).
24. Brunetti, A., Foti, D. & Goldfine, I. D. Identification of unique nuclear regulatory proteins for the insulin receptor gene, which appear during myocyte and adipocyte differentiation. J. Clin. Invest. 92, 1288–1295 (1993).
25. Goldfine, I. D. The insulin receptor: molecular biology and transmembrane signalling. Endocr. Rev. 8, 235–255 (1987).
26. Kahn, C. R. The molecular mechanisms of insulin action. Annu. Rev. Med. 36, 429–451 (1985).
27. Czech, M. P. The nature and regulation of insulin receptor: structure and function. Annu. Rev. Physiol. 47, 357–381 (1985).
28. Lewitt, M. S., Denyer, G. S., Cooney, G. J. & Baxter, R. C. Insulin-like growth factor binding protein-1 modulates blood glucose levels. Endocrinology 129, 2254–2256 (1991).
29. Rajkumar, K., Krsek, M., Dheen, S. T. & Murphy, L. J. Impaired glucose homeostasis in insulin-like growth factor binding protein-1 transgenic mice. J. Clin. Invest. 98, 1818–1825 (1996).
30. Chiefari, E. et al. The CAMP-HMGA1-RBP4 system: a novel biochemical pathway for modulating glucose homeostasis. BMC Biol. 7, 24 (2009).
31. Dey, D. et al. Inhibition of insulin receptor gene expression and insulin signalling by fatty acid: interplay of PKC isoforms therein. Cell. Physiol. Biochem. 16, 217–228 (2005).
32. Chieffari, E. et al. Functional variants of the HMGA1 gene and type 2 diabetes mellitus. JAMA 305, 903–912 (2011).
33. Chieffari, E. et al. Pseudogene-mediated posttranscriptional silencing of HMGA1 can result in insulin resistance and type 2 diabetes. Nat. Commun. 1, 40 (2010). doi:10.1038/ncomms1040.
34. Desvergne, B., Michalik, I. & Wahli, W. Transcriptional regulation of metabolism. Physiol. Rev. 86, 465–514 (2006).
35. Czech, M. P. Insulin’s expanding control of forkheads. Proc. Natl. Acad. Sci. USA 100, 11198–11200 (2003).
36. Puig, O. & Tjian, R. Transcriptional feedback control of insulin receptor by dFOXO/FOXO1. Genes Dev. 19, 2433–2446 (2005).
37. Paonessa, F. et al. Activator protein-2 overexpression accounts for increased insulin receptor expression in human breast cancer. Cancer Res. 66, 5085–5093 (2006).
38. Vitiellos, V. et al. Perturbations in homocysteine-linked redox homeostasis in a murine model for hyperhomocysteinemia. Am. J. Physiol. Regul. Integr. Comp. Physiol. 287, R39–R46 (2004).
39. Gerrish, K., Cissell, M. A. & Stein, R. The role of hepatic nuclear factor 1a and PDX-1 in transcriptional regulation of the pdx-1 gene. J. Biol. Chem. 276, 47775–47784 (2001).

Acknowledgments
We express our gratitude to Dr L. Levintow for critical reading of the manuscript. We also thank Dr I.D. Goldfine for helpful discussion and suggestions, and F.S. Brunetti for his help in artwork preparation. This work was supported by Telethon-Italy, grant GGP04245, and MIUR, protocol 2004062059-002 Italy, to A. Brunetti.

Author contributions
E.C. participated in the analysis and discussion of the data and drafting of the manuscript; M.N. and C.P. performed photomobilization 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

Competing financial interests: The authors declare no competing financial interests.
License: This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivative Works 3.0 Unported License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-nd/3.0/

How to cite this article: Chiefari, E. et al HMGA1 is a novel downstream nuclear target of the insulin receptor signaling pathway. Sci. Rep. 2, 251; DOI:10.1038/srep00251 (2012).