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GAS, a new glutamate-rich protein, interacts differentially with SRCs and is involved in oestrogen receptor function

Jing Liang, Hua Zhang, Yu Zhang, Ying Zhang & Yongfeng Shang[+]

Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Biochemistry and Molecular Biology, Peking University Health Science Center, Beijing, China

Steroid receptor coactivators (SRCs) exert profound effects on animal development and physiology. Genetic ablation experiments indicate that various SRC proteins might have differential physiological roles; however, clear evidence of functional specificity has not yet been shown at the molecular level. Here we report the identification of a new SRC1 interacting protein, glutamate-rich coactivator interacting with SRC1 (GAS), which contains a central glutamate-rich region and has transactivation activity. Interestingly, GAS interacts only with SRC1, and not with glucocorticoid receptor interacting protein 1 (GRIP1) or amplified in breast cancer 1 (AIB1), the other two members of the SRC family. It interacts with oestrogen receptor-α (ERα) and participates in both oestrogen receptor-regulated gene transcription and oestrogen-stimulated G1/S cell-cycle transition. Our data thus indicate that GAS is a new transcription cofactor and that different SRCs are associated with distinct secondary cofactors.

Keywords: coactivator; oestrogen receptor; transcription; breast cancer

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INTRODUCTION

Transcriptional activation is caused by transcription factors acting together with various coactivators and coregulators. The steroid receptor coactivators (SRCs or p160 coactivators) were initially described as transcription coactivators for nuclear receptors; however, they have since been found to also coregulate transcription initiated by other transcription factors such as AP1 (activator protein 1), STATs (signal transducers and activators of transcription), ETS (E26 transformation-specific factor), p53, E2F (E2 promoter binding factor) and NF-κB (Lonard & O'Malley, 2006, 2007). Many—if not most—animal genomes contain a set of three paralogous SRC genes that are highly conserved across species. In humans, the three SRC genes have been given the official names nuclear receptor coactivator 1 (*NCOA1*; also known as *SRC1*), *NCOA2* (*GRIP1*, *TIF2* or *SRC2*) and *NCOA3* (*AIB1*, *SRC3*, *pCIP*, *ACTR*, *RAC3* or *TRAM1*; Glass & Rosenfeld, 2000).

Two factors contribute to the ability of SRC proteins to function as transcriptional coactivators: an intrinsic histone acetyltransferase (HAT) activity (Spencer *et al*, 1997) and, more importantly, an ability to recruit multiple secondary or downstream cofactors into functional complexes (Lonard & O'Malley, 2007). Structurally, the three human SRC proteins share 40% identity in primary sequence and contain several signature domains important for their biological functions. The amino-terminal basic helix–loop–helix/Per-Arnt-Sim (bHLH-PAS) domain is important for DNA-binding specificity and for recruiting secondary coactivators such as GAC63, CoCoA and Fli-I (Kim *et al*, 2003; Lee *et al*, 2004; Chen *et al*, 2005). Additional domains include the central nuclear receptor-binding motifs (LXXLL, where L is leucine and X is any amino acid) and the two carboxy-terminal activation domains (ADs) (Heery *et al*, 1997; Sheppard *et al*, 2001). AD1 recruits proteins with strong HAT activity (such as CBP and p300), and AD2 interacts with the histone arginine methyltransferases CARM1 and PRMT1 (The Breast Cancer Linkage Consortium, 1999; Chen *et al*, 1999; Leo *et al*, 2000).

The physiological functions and pathological potentials of the SRC proteins, both during development and in adult life, have been studied extensively. Significantly, different *SRC* knockouts lead to distinct phenotypes (Xu *et al*, 1998, 2000; Wang *et al*, 2000; Gehin *et al*, 2002; Picard *et al*, 2002); however, little is known about the specific biological activities of individual SRC proteins at the molecular level. Most *in vitro* studies and transfection experiments indicate that each member of the SRC family is able to interact with several nuclear receptors and that a particular nuclear receptor can interact with all three members of the SRC family, leaving open the question of whether distinct intracellular SRC partnerships might specify particular phenotypic behaviours.

Here, we used genome-wide screening to search for proteins that preferentially interact with specific members of the SRC family, hoping to uncover specific biological activities for such

Department of Biochemistry and Molecular Biology, Peking University Health Science Center, 38 Xue Yuan Road, Beijing 100191, China
[+]Corresponding author. Tel: +86 10 82805118; Fax: +86 10 82801355;
E-mail: yshang@hsc.pku.edu.cn

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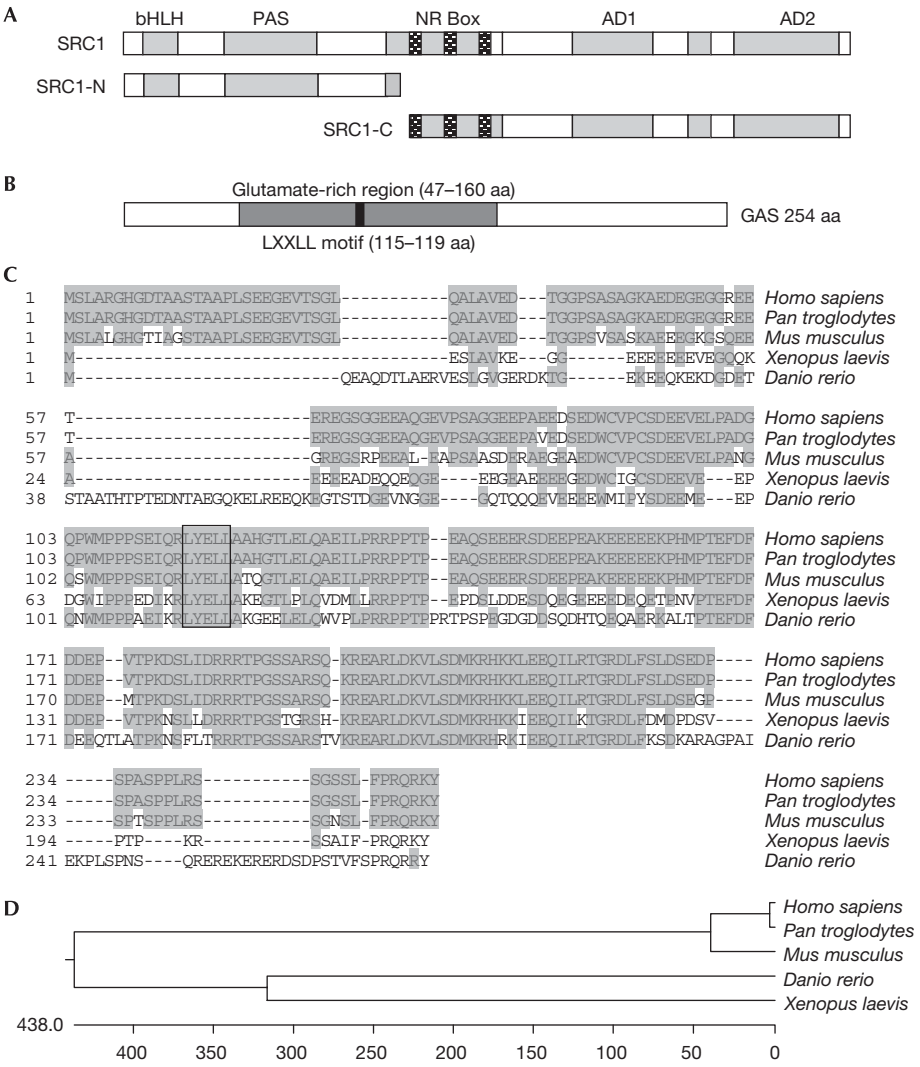

Fig 1 | Cloning and characterization of glutamate-rich coactivator interacting with SRC1. (**A**) Schematic representation of the structure and deletions of steroid receptor coactivator (SRC) 1. (**B**) Schematic representation of the protein structure of GAS. (**C**) Amino-acid sequence alignment of GAS from different species. The shaded area represents the conserved region and the LXXLL motif is boxed. (**D**) Phylogenetic analysis of the evolutionary relationships among GAS proteins from different species. AD, activation domain; bHLH, basic helix–loop–helix; C, carboxyl terminus; GAS, glutamate-rich coactivator interacting with SRC1; N, amino terminus; NR, nuclear receptor; PAS, Per-Arnt-Sim.

partnerships. We identified a new protein, glutamate-rich coactivator interacting with SRC1 (GAS), which preferentially interacts with SRC1 but not with the other two SRC proteins. We show that GAS behaves as a transcription coactivator and participates in oestrogen receptor (ER) functions.

RESULTS AND DISCUSSION

The three members of the SRC family—SRC1, GRIP1 and AIB1—contain several important functional domains, which often act as scaffold elements in the formation of higher-order regulatory complexes. In an effort to identify proteins that interact with specific members of the SRC family and thereby define their unique biological activities, we performed yeast two-hybrid experiments using the N-terminal fragments of the SRC proteins as bait to screen against a human mammary cDNA library (Zhang *et al*, 2006, 2007; Fig 1A). A clone match to the 3′ end (bases 259–780) of an open reading frame of gene *C16orf53* (GenBank accession number NM_024516) was recovered using SRC1-N bait. Back-hybridization experiments confirmed an interaction between this clone and SRC1, but not between GRIP1 and AIB1 (data not shown). We cloned the entire open reading frame of the *C16orf53* gene and named the protein 'glutamate-rich coactivator associated with SRC1 (GAS), as it contains a central glutamate-rich region (GAS) (Fig 1B). GAS is evolutionarily conserved across species (Fig 1C,D), and ubiquitously expressed in several human tissues and cell lines (Fig 2A and supplementary Fig 1A–C online). Immunostaining of endogenous GAS in MCF-7 cells revealed that the protein is largely localized to the nucleus, whereas over-expressed GAS is localized to both the cytoplasm and nucleus (supplementary Fig 1D online).

The direct interaction between GAS and SRC1 was confirmed by both *in vivo* immunoprecipitation (Fig 2B,C; note that the

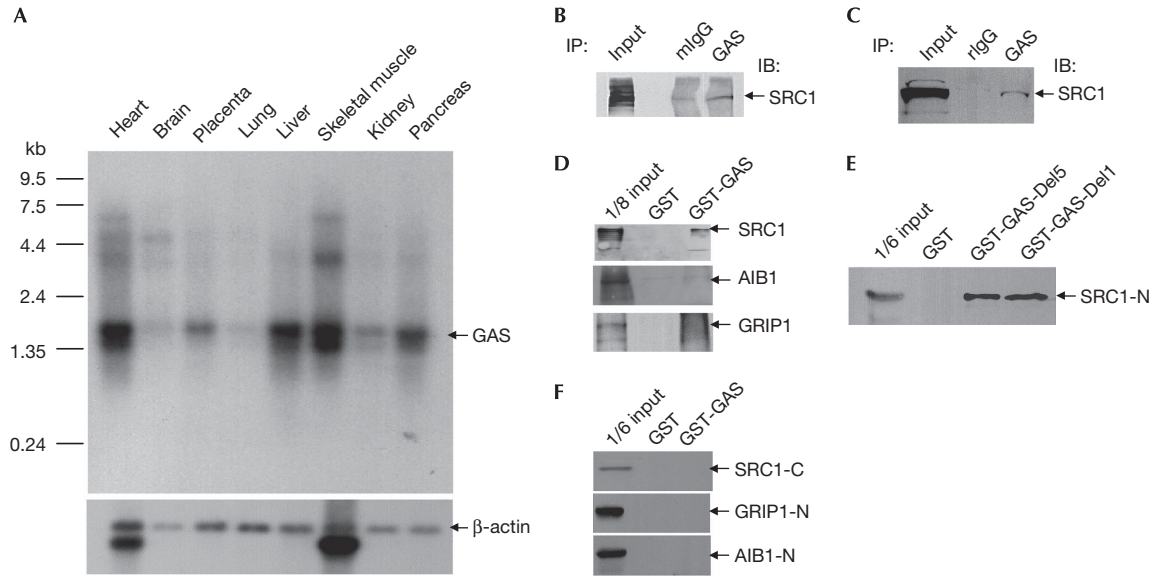

Fig 2 | The expression of glutamate-rich coactivator interacting with SRC1 (GAS) in human tissues, and the direct interaction between GAS and steroid receptor coactivator 1 (SRC1). (**A**) Northern blot analysis of GAS expression in human tissues. Using the entire coding sequence as a probe, Northern blotting showed a principal transcript of around 1.8 kb. The heart, liver and skeletal muscle show comparatively high expression of the principal transcript and, on the basis of results from these tissues, further (longer) isoforms also seem to exist. (**B**) MCF-7 cells were transfected with FLAG-GAS and SRC1 constructs. At 48 h after transfection, cell lysates were collected and either control IgG (mouse) or monoclonal FLAG antibodies were used to precipitate GAS-containing protein complexes. The precipitates were blotted with monoclonal SRC1 antibody. (**C**) 2×10^7 ECC-1 cells (which express a higher amount of SRC1 (Shang & Brown, 2002; Wu *et al*, 2005)) were used to detect the interaction between endogenous GAS and SRC1. Six micrograms of either control IgG (rabbit) or affinity-purified polyclonal GAS antibodies were used to precipitate endogenous GAS-containing protein complexes. (**D**) Glutathione *S*-transferase (GST) pull-down assays show that GAS interacts directly with SRC1 *in vitro*. The top panel shows a GST pull-down assay carried out with *in vitro* translated SRC1 and purified GST protein or GST-GAS protein. The two other panels show similar assays carried out with *in vitro* translated AIB1 or GRIP1. Three milligrams of GST-GAS protein was used in the upper panel, whereas 10 mg GST-GAS protein was used in the lower two panels. (**E**) GST pull-down assays confirm that GAS interacts strongly with the N-terminal region of SRC1. As the polyclonal SRC1 antibody used for the N-terminal region of SRC1 recognizes a nonspecific band of GST-GAS, which runs at almost the same position as SRC1-N, we used two deletion mutants of GST-GAS (Del-5 and Del-1, as in Fig 3D) to avoid the interference. (**F**) GAS does not interact with the C-terminal region of SRC1, or the N-terminal regions of GRIP1 or AIB1. SRC1-C was detected by a monoclonal SRC1 antibody, and GRIP1-N or AIB1-N were detected by a monoclonal Myc antibody IB, immunoblotting; IP, Immunoprecipitation.

endogenous co-immunoprecipitation assay in Fig 2C was carried out in ECC-1 cells due to higher levels of SRC1 in these cells) and *in vitro* glutathione *S*-transferase (GST) pull-down assays (Fig 2D). Consistent with our original yeast two-hybrid results, the region responsible for binding in SRC1 was located in the N-terminal and not in the C-terminal (Fig 2E,F). Interestingly, when we examined whether GAS would also interact with GRIP1 or AIB1, we found that, although 3 μg of GST-GAS pulled down SRC1 effectively, as much as 10 μg of GST-GAS failed to pull down any detectable AIB1 or GRIP1 (Fig 2D). Furthermore, GST-GAS was also unable to pull down the N-terminal part of AIB1 or GRIP1 (Fig 2F). This corroborates the results of our yeast two-hybrid experiments and again supports the observation that GAS preferentially binds to SRC1 but not the other two SRC proteins.

Many intracellular SRC partners, including human MMS19 (methyl methanesulfonate-sensitive 19; Wu *et al*, 2001), TIF1α (transcriptional intermediary factor 1; Teyssier *et al*, 2006), Fli-I (Flightless I; Lee *et al*, 2004) and GAC63 (GRIP1-associated coactivator 63; Chen *et al*, 2005), can interact with nuclear receptors. A specific binding of ERα to GAS both *in vivo* and *in vitro* was detected by immunoprecipitation (Fig 3A) and GST pull-down assays (Fig 3B). Similar to other nuclear receptors, ERα contains several characteristic domains: an N-terminal ligand-independent activation domain (AF1), a DNA-binding domain, a hinge region, and C-terminal ligand-binding (LBD) and activation (AF2) domains (Fig 3C). GAS was shown to bind to the N-terminal AB region of ERα (AF1 domain, Fig 3C), which is consistent with the ligand-independent characteristic observed in our immunoprecipitation and GST pull-down assays. GAS does not interact with RARα (retinoic acid receptor α), another nuclear receptor (supplementary Fig 2B online), suggesting that the interaction between GAS and ERα is relatively specific. By making systematic GAS deletion mutants, we mapped the region in GAS responsible for binding to ERα or SRC1 (Fig 3D). The C-terminal part (from 161 to 254 aa) of GAS is sufficient to bind to ERα, whereas the GAS middle region (from 116 to 160 aa), including the LXXLL motif, is responsible for its interaction with SRC1. It is interesting that the LXXLL motif (LYELL) in GAS is not crucial for binding to ERα, but seems be important in binding to SRC1 (Fig 3D middle-right panel, compare GST-GAS Del-5 and GST-GAS Del-6).

The presence of the central acidic (glutamate-rich) region suggests that GAS could be involved in transcription activation

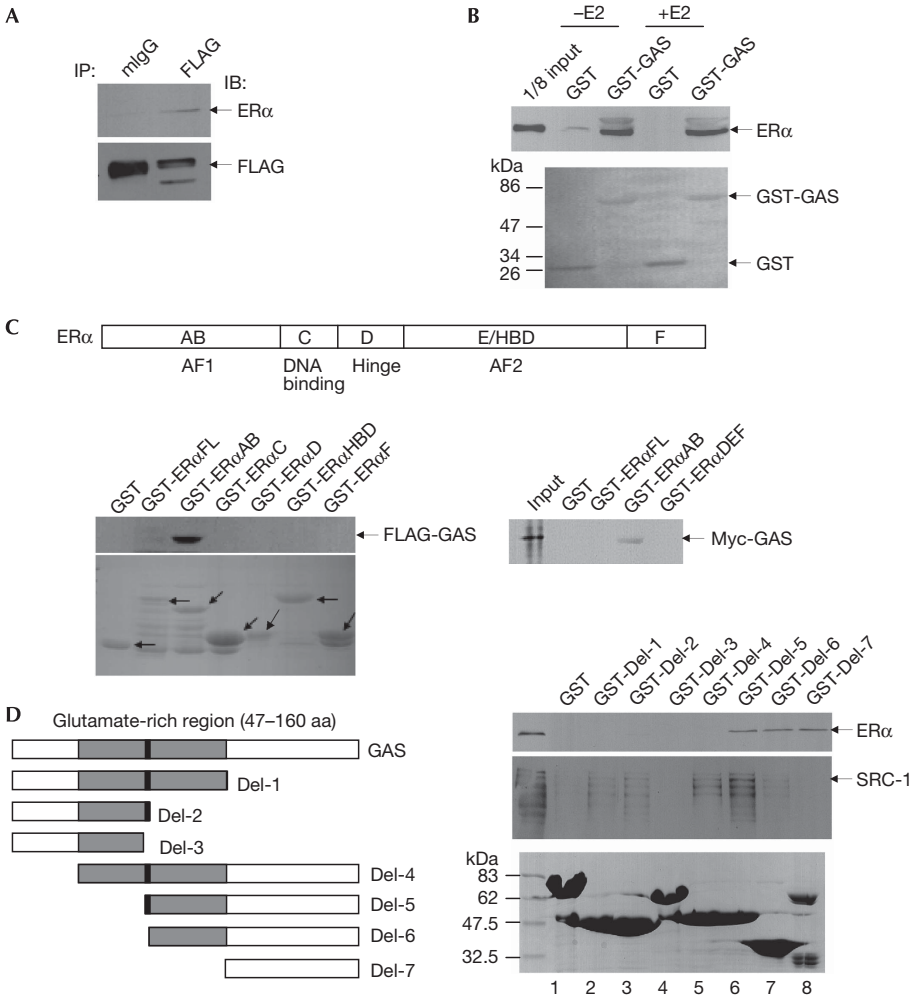

Fig 3 | The direct interaction between glutamate-rich coactivator interacting with SRC1 and oestrogen receptor-α. (**A**) *In vivo* immunoprecipitation (IP) of overexpressed FLAG-GAS and ERα. MCF-7 cells were transfected with a FLAG-GAS construct and subjected to co-immunoprecipitation (co-IP) assays after 48 h, similar to that described in Fig 2A. The precipitates were then analysed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) with polyclonal ERα antibody. (**B**) Glutathione *S*-transferase (GST) pull-down assay shows that GAS interacts directly with ERα *in vitro*. Three milligrams of either purified GST or GST-GAS protein immobilized on glutathione sepharose 4B beads was incubated with *in vitro* translated ERα in the presence (+) or absence (−) of 100 nM E2 for 2 h. After extensive washing, the beads were subjected to SDS–PAGE and probed with polyclonal ERα antibody (the upper panel). The lower panel is a Ponceau staining of the purified proteins. (**C**) A schematic drawing of the ERα protein. The respective domains, AB (1–184 aa), C (185–250 aa), D (251–314 aa), E (315–540 aa) and F (541–596 aa), were fused to GST to generate the ERα deletion mutants (lower-left panel; Coomassie staining). GST pull-down experiments were performed with *in vitro* translated FLAG-GAS and purified GST or different GST-ERα deletion mutants; Western blot using monoclonal FLAG antibody was used to examine the precipitated complexes. Full-length (FL) ERα pulled down little FLAG-GAS, probably because of degradation in the stored GST-ERα protein. To exclude the improper folding of the E region in the absence of surrounding amino acids, the DEF region was also fused to GST, and GST pull-down experiment was repeated (lower right panel). *In vitro* translated Myc-GAS was used this time and a monoclonal Myc antibody was used to examine the precipitates. (**D**) A series of GAS deletion mutants were fused to GST to map the domains responsible for interactions with ERα and steroid receptor coactivator (SRC) 1. The left panel shows a schematic drawing of the mutants, and the lower right panel shows Coomassie blue staining of the purified proteins (lanes 1–7 denote GAS-Del-1 to GAS-Del-7, respectively; lane 8 represents the purified GST-ERα-DEF protein). The two upper-right panels show GST pull-down results using the GAS mutants and *in vitro* translated ERα or SRC1. ER, oestrogen receptor-α; GAS, glutamate-rich coactivator interacting with SRC1.

(Kim *et al*, 2006; Titz *et al*, 2006; Zhao *et al*, 2006). This possibility was first confirmed in a GAL4 reporter assay system. Compared with the control GAL4-DBD (DBD for DNA binding domain), the expression of GAS-GAL4-DBD resulted in an increase in GAL4-TK-luciferase reporter gene transcription, indicating that GAS can enhance gene transcription when it is arbitrarily recruited to target promoter (Fig 4A). The direct interaction between GAS and ERα, and between GAS and SRC1, prompted us to examine further whether GAS is involved in oestrogen receptor-mediated transcriptional regulation. Overexpression of GAS in MCF-7 cells is indeed associated with an increase in oestrogen receptor-responsive element luciferase reporter activity (Fig 4B), whereas

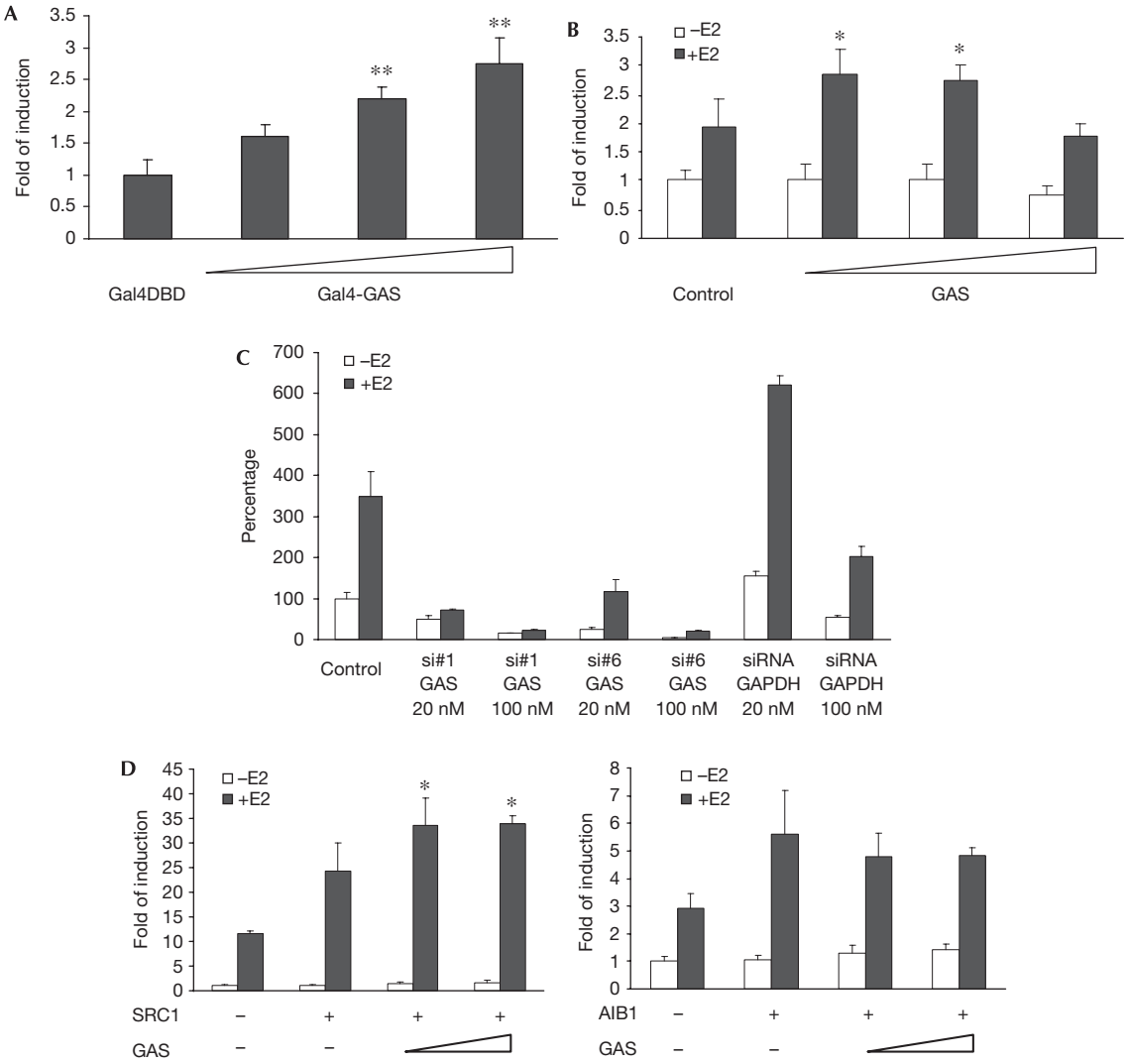

Fig 4 | Glutamate-rich coactivator interacting with SRC1 has autonomous transactivation activity and is required for oestrogen receptor-mediated transactivation. (**A**) The entire GAS open reading frame was cloned downstream from the DNA-binding domain of Gal4 (Gal4-DBD), and either the resulting construct or a control vector (containing Gal4-DBD only) was cotransfected with 100 ng of a Gal4-TK-luciferase reporter construct into MCF-7 cells. Increasing amounts of Gal4-DBD-GAS (100, 200 and 600 ng) or 600 ng Gal4-DBD constructs were used and total plasmid DNAs were made up to 800 ng with empty pcDNA3.1 vectors. Cells were collected 24 h after transfection, and luciferase activity was measured and normalized to that of renilla. (**B**) MCF-7 cells were grown in medium lacking oestrogen (E2) for 24 h and cotransfected with increasing amounts of pcDNA3.1-GAS construct (100, 200 and 400 ng) and 100 ng 6 × ERE-TK-luciferase reporter construct; cells were allowed to continue growing for 24 h before adding (or not adding) 100 nM of E2. Cell lysates were collected after 24 h of E2 treatment, and luciferase activity was measured and normalized to that of renilla. (**C**) Knockdown of GAS expression in MCF-7 cells significantly affected the oestrogen receptor-mediated reporter gene activation. MCF-7 cells were deprived for E2 for 24 h and grown to about 30% confluence. Cells were then transfected with synthesized short interfering RNAs (siRNAs) against GAS or GAPDH, together with 100 ng of 6 × ERE-TK-luciferase reporter vector. Cells were incubated for a further 24 h before E2 treatment as in (**B**). (**D**) CV-1 cells were transfected with 6 × ERE-TK-luciferase reporter plasmid (100 ng), pHE0 encoding human oestrogen receptor (2 ng), pcDNA3.1/steroid receptor coactivator (SRC) 1 (400 ng) and pcDNA3.1/GAS (100 or 200 ng) as indicated, and grown in a medium containing or lacking E2. The results shown are representative of three independent experiments. The right panel was done similarly, except that pcDNA3.1/AIB1 (400 ng) was used instead of the SRC1 construct. Student's *t*-test was used for statistical analysis. $*P<0.05$ and $**P<0.001$. ER, oestrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAS, glutamate-rich coactivator interacting with SRC1.

knockdown of GAS expression by RNA-mediated interference resulted in considerable inhibition of reporter activity (Fig 4C). Collectively, these experiments suggest that GAS enhances oestrogen receptor-regulated gene transcription.

GAS interacts with the N-terminal bHLH-PAS domain of SRC1, similar to several so-called secondary coactivators such as GAC63 (Chen *et al*, 2005), CoCoA (Kim *et al*, 2003) and Fli-I (Lee *et al*, 2004). These proteins generally enhance oestrogen receptor-mediated gene transcription in a manner that is strictly dependent on interaction with SRCs. However, some of them—such as GAC63 and Fli-I—also interact with ERα directly, although it is not clear to which region in ERα they actually bind (Lee *et al*, 2004;

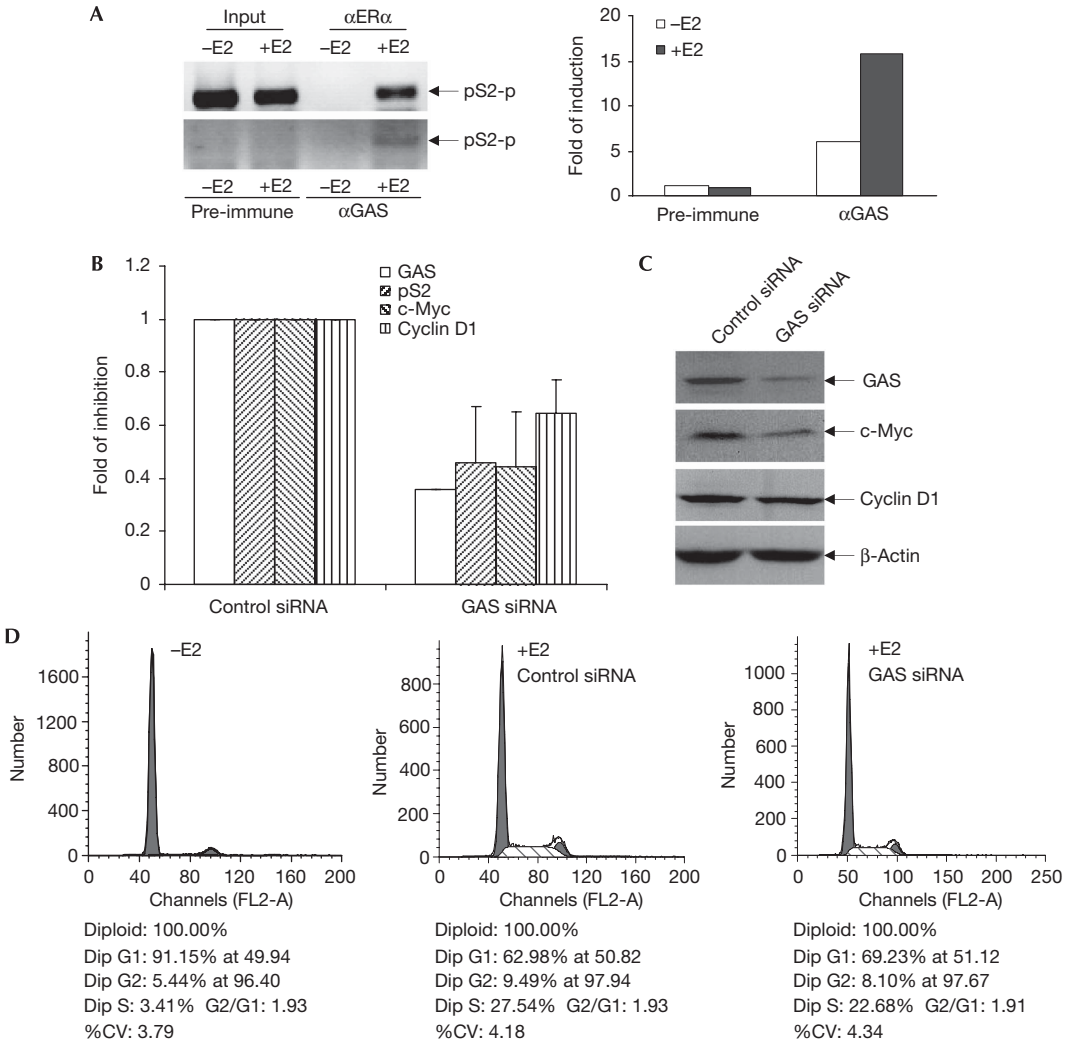

Fig 5 | Glutamate-rich coactivator interacting with SRC1 is required for oestrogen function. **(A)** ChIP assay shows that endogenous GAS is recruited to the promoter region of pS2 (−353 to −30 bp upstream from the transcription initiation site), a classic oestrogen receptor (ER) target gene. The primers for pS2 promoter region were described previously (Zhang *et al*, 2004). **(B)** Endogenous GAS expression in MCF-7 cells under normal culture conditions was knocked down using 100 nM GAS short interfering RNA (siRNA) 6 (which was used for A, B and C), and endogenous levels of pS2, c-Myc and cyclin D1 were analysed using real-time RT–PCR. The results shown here are the averages of at least three independent experiments. **(C)** Reduced amounts of GAS expression led to reduced amounts of c-Myc and cyclin D1 protein, as shown by Western blotting. **(D)** A fluorescence-activated cell sorting analysis showing that GAS-directed siRNA, but not scrambled siRNA, impairs normal cell-cycle progression through the G1/S transition in MCF-7 cells. The results shown here are representative of three independent experiments. ChIP, chromatin immunoprecipitation; GAS, glutamate-rich coactivator interacting with SRC1.

Chen *et al*, 2005). In a well-documented transcription synergy testing system (Kim *et al*, 2003; Lee *et al*, 2004), GAS and SRC1 together produce an effect on reporter transcription that is at least additive, whereas no synergy or additive effect was observed with GAS and AIB1 (Fig 4D). A chromatin immunoprecipitation (ChIP) assay showed that endogenous GAS is present in the promoter region of pS2, a classic oestrogen receptor target gene (Fig 5A), indicating that GAS is involved in the transcriptional regulation of endogenous oestrogen receptor target genes. Consistent with the above observations, depletion of GAS by short-interfering RNA in MCF-7 cells cultured in normal conditions led to a reproducible decrease in messenger RNA (Fig 5B) and protein levels (Fig 5C) of three representative oestrogen receptor target genes: pS2, c-Myc and cyclin D1. GAS (referred to as PA1) was recently found to be associated with a SET1-like methyltransferase complex specific for H3K4 methylation (Cho *et al*, 2007), which generally marks transcription activation. Although it is possible that the coactivator activity of GAS is related to H3K4 methylation status, knockdown of GAS only slightly decreased H3K4 methylation in the pS2 promoter (supplementary Fig 4 online). Finally, as oestrogen is normally required for the G1/S transition of MCF-7 cells (Doisneau-Sixou *et al*, 2003; Shang, 2006; Shi *et al*, 2007), fluorescence-activated cell sorting analysis indicated that knockdown of GAS expression impeded the oestrogen-stimulated G1/S transition (Fig 5D) in these cells, supporting a role for GAS in oestrogen function at whole-cell level.

In summary, our study suggests that GAS interacts directly with SRC1 and ERα, and enhances ERα-related gene transcription regulation. Together with SRC1, GAS might also participate in other transcription pathways such as p53 (supplementary Fig 2A,C online); the overall biological function of GAS remains to be investigated further. Interestingly, despite the high degree of similarity among the three SRC proteins, GAS seems to interact only with SRC1—not with GRIP1 or AIB1. This specific binding between SRC1 and GAS is possibly because of the potential unique post-translational modifications of SRC1 (supplementary Fig 3 online; SRC1-N has potential unique serine phosphorylation sites). Differential post-translational modifications might alter the three-dimensional structure of individual SRC homologues, which further recruit their specific intracellular partners. As most SRC partners studied so far have been found to interact with all the three SRC proteins indiscriminately, the preference of GAS for SRC1 might offer a new mechanism for cells to fine-tune the expression of different SRC target genes.

METHODS

For the yeast two-hybrid experiments, the N-terminal fragment (1–1896 bp) of SRC1 was fused to a BD (GAL4 DNA binding domain) plasmid and used as bait, in screening a human mammary gland cDNA library. The Matchmaker GAL4 Two-Hybrid System 3 (Clontech protocol PT3247-1) was used as described by Zhang *et al* (2006, 2007). To clone the complete GAS open reading frame, total RNA was extracted from human endometrial cancer cell line Ishikawa cells for use as a template, and RT–PCR was performed using GAS-specific primers. The resulting fragment was cloned into pcDNA3.1(−) vectors and sequenced to confirm the coding sequence. This sequence was found to match the CDS of NM_024516 exactly. The conserved domains and motifs of GAS were predicted from the database at www.expasy.ch/tools. Multiple alignments were performed using ClustalW (version 1.60), and phylogenetic analysis using the Jotun Hein method.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org)

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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