# Supplementary material for:

**Iron-responsive degradation of iron regulatory protein 1**

**does not require the Fe-S cluster:**

**S.L. Clarke, et al.**

## Materials and Methods:

*Fe-S Cluster Reconstitution:* Cells treated with 50 μM desferal for 18 hr were lysed in an anaerobic chamber (< 10 ppm $O_2$). Anaerobic lysis buffer B contained 20 mM Tris-HCl pH 7.5, 300 mM NaCl, 1% Triton X-100, 1 mM citrate, 10 mM EDTA and protease inhibitors as described above. Cells were lysed as described (Eisenstein et al., 1993) except 4 volumes buffer B was used. The clarified lysate was stored in liquid nitrogen. Reconstitution reactions (100 μl) contained cell lysate (0.4 to 0.7 mg protein), 1 mM $Fe^{2+}$ (ferrous ethylene diammonium sulfate, GFS Chemicals), 1 mM L-Cys, 8 mM DTT, and 0.06 mg/ml NifS protein. Reactions proceeded anaerobically for 2 hr at room temperature.

## Results:

### S138 Phosphomimetic Mutants of IRP1 Display Impaired Accumulation as c-Acon:

After tet induction the spontaneous RNA binding activities of $IRP1^{WT}$, $IRP1^{S138A}$, and $IRP1^{S138E}$ were 1.8, 1.2 and 2.9 pmol RNA bound/mg lysate protein, respectively (**Supplementary Figure 1A**). Spontaneous binding refers to the IRP1 binding activity present in cell extracts when the cells are lysed (i.e. before addition of high levels of 2-ME) and is viewed as the free pool of IRP1 available for binding to RNA in the cell. However, S138E protein was only about 30% as

abundant as $IRP1^{WT}$ or $IRP1^{S138A}$ protein (**Supplementary Figure 1B**) (see legend for quantification of western data). Hence, when the RNA binding activities were normalized to the level of each IRP1 transgene, $IRP1^{S138E}$ exhibited an RNA binding capacity that was 5- and 7-fold higher than $IRP1^{WT}$ and $IRP1^{S138A}$, respectively (**Supplementary Figure 1C**).

We determined the size of the c-acon pool of IRP1 by gel shift with 2-ME and by aconitase assays. $IRP1^{WT}$ RNA binding activity was increased 3-fold by 2-ME (**Supplementary Figure 1D**), a response that reflected the 2-ME effect on endogenous IRP1 protein (results not shown). A similar 2-ME effect was observed for $IRP1^{S138A}$ as was seen for $IRP1^{WT}$ while a somewhat reduced response (2-fold) was noted for $IRP1^{S138E}$ (**Supplementary Figure 1D**). The ability of 2-ME to activate RNA binding by $IRP1^{WT}$ and S138 mutants indicates that a substantial portion of these proteins is present in the c-acon or other latent form (i.e. either the [3Fe-4S] holoprotein or cysteine-oxidized apoprotein, neither of which binds RNA well (Schalinske et al, 1997). To assess further the size and characteristics of the latent pool of IRP1, the effect of expressing $IRP1^{WT}$ and S138 mutants on cellular aconitase activity was determined. In the absence of tet, extracts of cells capable of expressing $IRP1^{WT}$, $IRP1^{S138A}$ or $IRP1^{S138E}$ all had similar endogenous aconitase activities of ~28 mU/mg protein (**Supplementary Figure 1E**). Following tet induction aconitase activity in lysates from cells expressing $IRP1^{WT}$ and $IRP1^{S138A}$ doubled to ~60 mU/mg protein. In contrast, there was no detectable change in aconitase activity in extracts of cells expressing $IRP1^{S138E}$ after tet induction (**Supplementary Figure 1E**) even though the $IRP1^{S138E}$ protein level was substantially induced (result not shown). Since tet induction of $IRP1^{S138E}$ gives a 2-fold increase in total IRP1 protein in HEK cells (results not shown) and a 2- to 4-fold increase in spontaneous IRP1 RNA binding activity a similar fold increase in aconitase activity had been anticipated. Consequently, it was then determined if

IRP1$^{S138E}$ expressed in HEK cells could be converted to an aconitase *in vitro*. After *in vitro* cluster reconstitution, extracts from tet-induced IRP1$^{S138E}$ cells had 2-fold more aconitase activity than did extracts from cells capable of expressing IRP1$^{S138E}$ but that had not been tet induced (**Supplementary Figure 1F**). The 2-fold increase over maximum endogenous aconitase activity for IRP1$^{S138E}$ indicates it can form a [4Fe-4S] cluster. Taken together these results argue that IRP1$^{S138E}$ phosphomimetic mutant of IRP1 preferentially accumulates in the RNA binding form at the expense of the c-acon form but this is not due to an inability of IRP1$^{S138E}$ to be an aconitase.

## IRP1 Can be Phosphorylated at Serine 138 by Protein Kinase C:

To further illustrate the physiological relevance of S138 phosphorylation, phosphorylation state-specific antibodies were developed. These antibodies recognize purified IRP1$^{WT}$ only after incubation with protein kinase C (PKC) (**Supplementary Figure 2A**, lane 2). Neither native nor PKC-treated IRP1$^{S138A}$ were recognized by the phosphospecific antibody (**Supplementary Figure 2A**, lanes 3 and 4). Total IRP1 protein level was the same in all lanes (**Supplementary Figure 2B**). To determine if S138 is phosphorylated in cultured cells, HEK cells expressing IRP1$^{WT}$ or IRP1$^{S138A}$ were treated with or without phorbol 12-myristate 13-acetate (PMA), a PKC activator. IRP1 was immunoprecipitated using the myc-tag and detected with S138 phosphospecific antibodies or pan-IRP1 antibodies. IRP1$^{WT}$, but not IRP1$^{S138A}$, was detected with the phosphospecific antibody in PMA treated cells (**Supplementary Figure 2C**, compare lanes 2 and 4). The total level of IRP1$^{WT}$ or IRP1$^{S138A}$ protein was not altered by PMA (**Supplementary Figure 2D**). When more protein was examined, S138-phosphorylated protein could be detected

in untreated cells (see **Supplementary Figureure 3**). These results indicate that S138 is an physiological phosphorylation site in IRP1.

## IRP1 is Phosphorylated at S138 in Untreated HEK Cells:

A S138 phosphoblot was run using extracts from untreated and 2 hr PMA (1 μM) treated cells. A low level of S138 phosphorylated protein was observed in untreated cells which increased after PMA treatment (**Supplementary Figure 3**, compare lanes 1 and 2). A second experiment involved incubating HEK 293 cells expressing $IRP1^{WT}$ with [$^{32}$P]orthophosphate for 4 hrs. IRP1 was immunoprecipitated using the c-myc monoclonal antibody 9E10 and analyzed by 2-D gel electrophoresis (**Figure 3C, main paper**). We conclude that IRP1 is phosphorylated at S138 in untreated cells.

## Supplementary Figure Legends:

**Figure 1: Phosphomimetic Mutants of IRP1 Accumulate in an RNA Binding Form in HEK 293 Cells. (A)** EMSA of non-transfected (lane 1) cells or cells expressing $IRP1^{WT}$ (lanes 2 and 3), $IRP1^{S138A}$ (lanes 4 and 5) and $IRP1^{S138E}$ (lanes 6 and 7). IRP1 RNA binding activities 1.8 ± 0.6, 1.2 ± 0.1 and 2.9 ± 0.5 pmol/mg lysate protein for cells expressing myc-tagged $IRP1^{WT}$, $IRP1^{S138A}$ or $IRP1^{S138E}$, respectively (mean ± SEM, n = 3). RNA binding for myc-tagged IRP1 was determined by supershift EMSA. **(B)** Immunoblot of IRP1 protein level in 2 independent clones, labeled A and B, of cells expressing myc-tagged $IRP1^{WT}$, $IRP1^{S138A}$ or $IRP1^{S138E}$ using anti-myc (9E10) antibodies. The protein level of $IRP1^{S138A}$ and $IRP1^{S138E}$, relative to $IRP1^{WT}$ are 90 ± 14 % and 32 ± 10 %, respectively. **(C)** RNA binding activity obtained by normalizing the

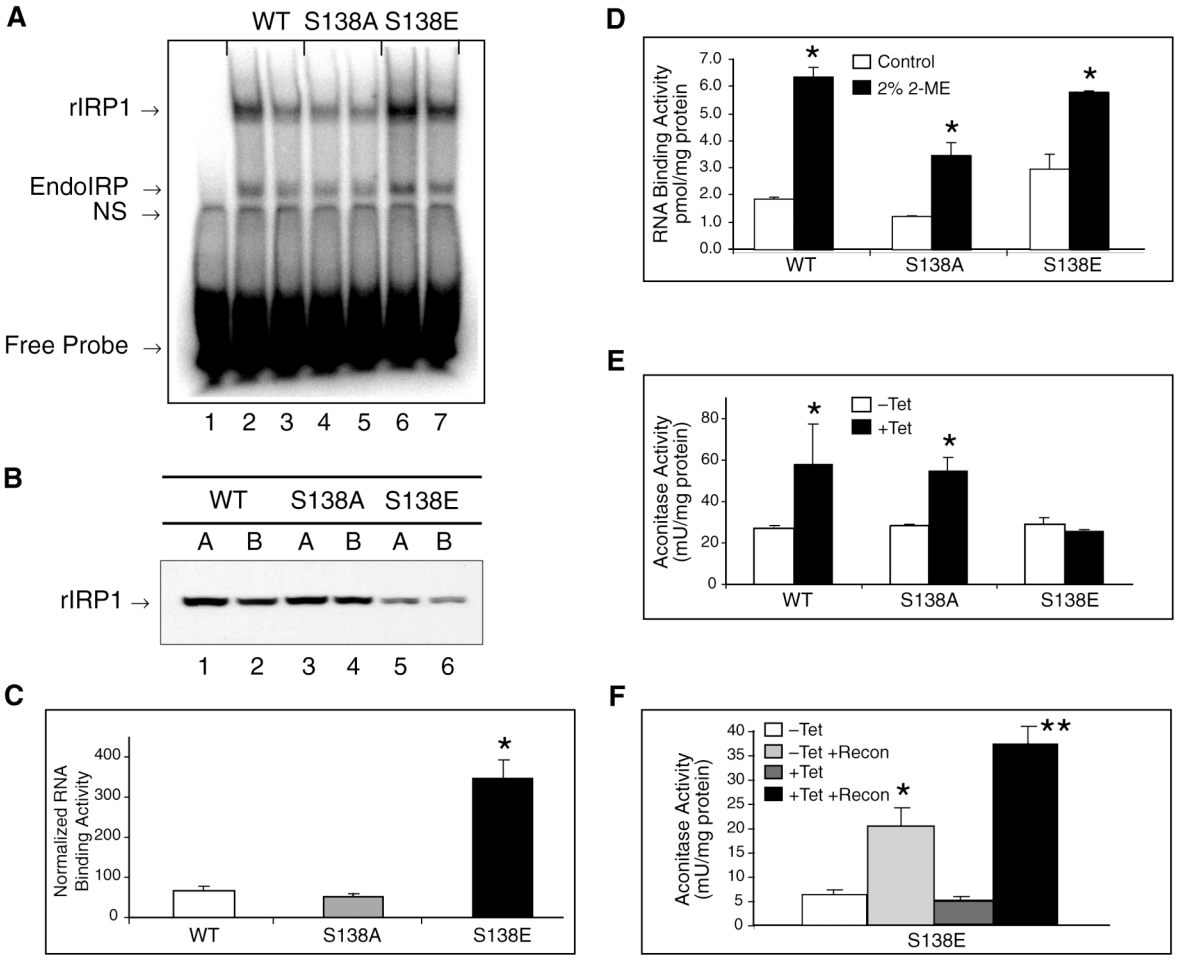
results from panel A by protein level in panel B. **(D)** Effect of 2% 2-ME on RNA binding activity of myc-tagged IRP1$^{WT}$, IRP1$^{S138A}$ or IRP1$^{S138E}$. (**E**) Aconitase activity in lysates was determined from cells that were not induced (-Tet) or induced (+Tet) with Tet for 36 hr. Immunoblot analysis showed induction of myc-tagged IRP1 proteins upon tet addition but no myc-tagged IRP1 was detected in the absence of tet (results not shown). (**F**) Cells capable of expressing myc-tagged IRP1$^{S138E}$ were incubated (36 hr) in the absence or presence of tet. Desferal was included in the medium to stabilize S138E protein. Cell extracts were obtained and then were or were not incubated under conditions to reconstitute the Fe-S cluster in IRP1 (see Methods). Results are mean ± SEM for n = 3. A similar increase in aconitase activity was noted in tet-treated cells expressing IRP1$^{WT}$ but not in non-transfected cells (results not shown). An asterisk indicates a significant difference from IRP1$^{WT}$ or IRP1$^{S138A}$ (panel C) or from control (panels D and E) ($P < 0.05$). For panel F one or two asterisks means significant difference relative to the respective no reconstitution control ($P < 0.05$) and two asterisks indicates +Tet + Recon different from –Tet +Recon ($P < 0.05$).

**Supplementary Figure 2: Serine 138 of IRP1 is a Phosphorylated *In vitro* and in HEK 293 Cells.**

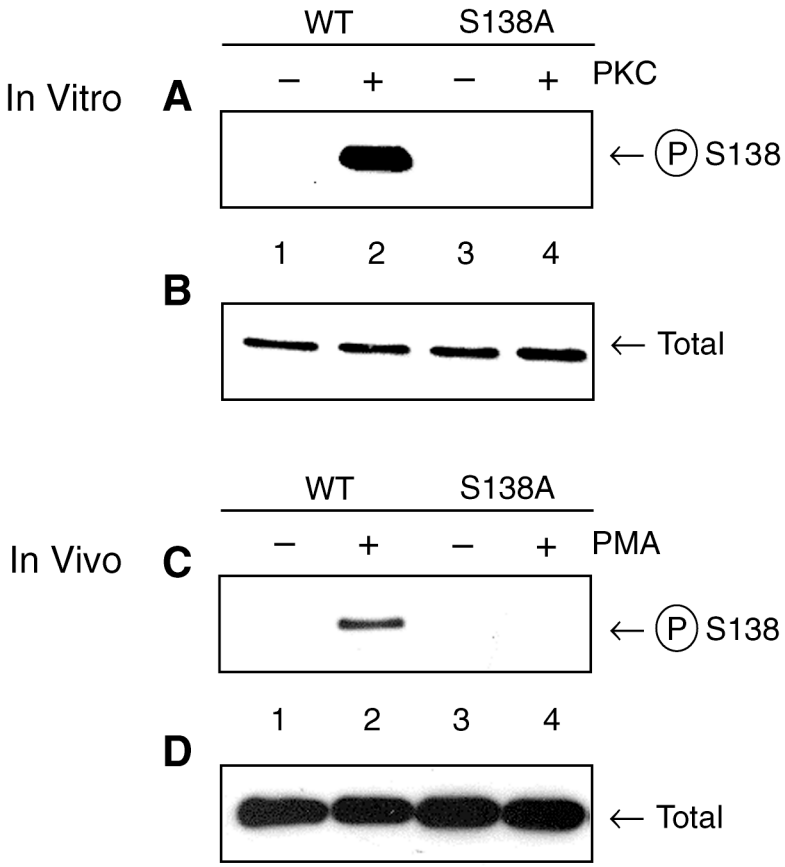
**(A** and **B) Purified** recombinant rabbit IRP1$^{WT}$ (lanes 1 and 2) or IRP1$^{S138A}$ (lanes 3 and 4) (56 ng protein) were treated without (lanes 1 and 3) or with (lanes 2 and 4) PKC as described (Eisenstein et al., 1993). (A) Immunoblot with S138 phosphospecific antibody. (B) Reprobing of panel A immunoblot with anti-rat liver IRP1 antibody. **(C and D)** HEK 293 cells expressing IRP1$^{WT}$ or IRP1$^{S138A}$ were treated with diluent (lanes 1 and 3) or 1 μM PMA for 1 hr (lanes 2 and 4). Cell lysate protein (500 μg) was immunoprecipitated and blotted. **(C)** Immunoblot with S138 phosphospecific antibody. **(D)** Same blot as panel C reprobed with non-phosphospecific antibody

against IRP1. S138 phosphorylated IRP1 was detected in untreated cells when a large amount (1 mg) of extract protein was used (data not shown).

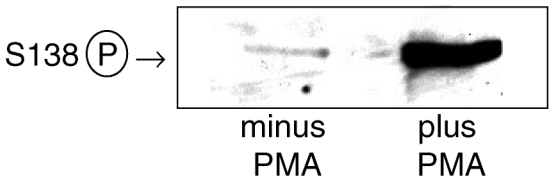
**Supplementary Figure 3: IRP1 is phosphorylated at S138 in untreated HEK 293 cells**. HEK 293 cells were treated with diluent (DMSO) or 1 μM PMA for 2 hr. After cell lysis myc-tagged IRP1 was immunoprecipitated from 1 mg of lysate protein that is twice the level of lysate protein used in panel C of supplementary figure 2.




Supplemental Figure 1




Supplemental Figure 2




Supplemental Figure 3