EXPRESSION OF ENDOPLASMIC RETICULUM OXIDOREDUCTASES (EROS) AND THEIR ROLE IN THE GI TRACT

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CHAPTER 5

ANALYSING POTENTIAL ERO1 MONOCLONAL ANTIBODIES

5.1 Introduction

As Ero proteins have different functional conformations and oxidative states, there is much value in being able to differentially detect these states *in vivo,* either in cell lysates, or in tissue samples. This is especially true for cells experiencing oxidative stress, which may dramatically alter the quality and output of secreted proteins, in which Eros play a major part through oxidative protein folding. At present, there is no published antibody specific to individual oxidative forms of Ero1α or Ero1β.

Having generated recombinant Ero1β fusion protein, this was used for monoclonal antibody production. Purified Ero1α, which the lab had previously used to generate the polyclonal antibody D5, was also taken along, together with plasmids encoding Ero1α for DNA immunisation. After immunisiation of naive mice and boosting of mice to produce a secondary immune response, the spleens were removed, washed in RPMI and a single cell suspension was made. Red blood cells were removed by lysis in a hypotonic buffer and the enriched splenocytes were fused with the murine myeloma cell line NSO. Cells were fused in a PEG/DMSO solution and the fused hybridomas were selected in HAT medium prior to plating out as single cell cultures. Surviving colonies were picked to determine whether they secreted monoclonal antibodies. Supernatants from cultures of these hybridomas were then generated, and tested using a prelimary ELISA assay in 24-well and 96-well plates (Benham and Knight, unpublished, 2009) to determine candidates for further testing in Western blots. The plates were designated 1.1-1.3 (from Ero1α protein immunised mice), 2.1-2.3 (from Ero1α C397A cDNA immunised mice), 3.1-3.3 (from Ero1β-HIS-GST protein immunised mice) and 4.1-4.6 (from Ero1β-HIS protein immunised mice). The use of plasmid cDNA has been used previously by Chang and colleagues to evoke an immune response to Japanese encephalitis in mice (Chang *et al*., 2000).

The ELISA assay plates resulted in a total of 61 monoclonal antibody supernatants that were further analysed in experiments shown in this chapter. The supernatants were screened using mouse and rat tissue lysates, mammalian Ero1α and Ero1β transfectants, and the Ero1β immunogen (where appropriate). It was expected that liver should give the strongest signal for Ero1α, whilst pancreas tissue would be a strong positive control for Ero1β, based on published expression profiles (Pagani *et al.*, 2000, Dias-Gunasekara *et al.*, 2005) and seen in datasets available through Pubmed GEO profiles.

5.2 Results

5.2.1 Supernatants

To simplify the description of the 61 supernatants taking for testing, the term "series" is used to denote the group of animals immunised. The numbers in each series represent individual hybridomas isolated by Dr A. Benham and Dr A. Knight.

Series 1: Ero1α wt immunised mice (9)

Series 2: Ero1α C397A cDNA immunised mice (14)

Series 3: Ero1β-HIS-GST protein immunised mice (10)

Series 4: Ero1β-HIS protein immunised mice (28)

The named supernatants used in the screening program are shown in Table 5.1.

Ero1α		Ero1β	
Series 1	Series 2	Series 3	Series 4
1.1-1.3	2.1-2.3	3.1-3.3	4.1-4.6
1.2A2	2.2A1	3.2B1	4.3A3
1.2B2	2.2A2	3.2D2	4.3A4
1.2B6	2.2A3	3.2D4	4.3B1
1.2D1	2.2B4	3.2D5	4.3B2
1.2D2	2.2C4 I	3.3A1	4.3B4
1.3B3	2.2C4 II	3.3A4	4.3B5
1.3C3	2.2C5	3.3A6	4.4A2
1.3C4	2.2C6	3.3C2	4.4A6
1.3D2	2.3B1	3.3C4	4.4B2
	2.3B2	3.3D2	4.4B5
	2.3B3		4.4B6
	2.3B6		4.4C6
	2.3C2		4.4D4
	2.3C4		4.5A1
			4.5A5
			4.5B1
			4.5B3
			4.5B5
			4.5C4
			4.5C5
			4.5D4
			4.5D5
			4.6A1
			4.6A3
			4.6A5
			4.6B5
			4.6C2
			4.6C3

Table 5.1 Supernatants tested by ELISA and used in antibody screening

A total of 61 supernatants were generated, for use in the antibody screening program. The 14 antibodies highlighted in green in the table were selected for re-testing, based on initial blot data.

5.2.1.1 Validation of murine and rat tissues

Male CD-1 mouse heart, liver, stomach and pancreas tissue lysates were initially used to screen supernatants by SDS-PAGE/Western blotting, alongside transfected cell lysates which were positive for Ero1α-myc and Ero1β-myc. All lysates were matched for protein concentration using the BioRad DC protein assay and stock lysates were prepared and snap frozen. In later experiments where potential Ero1α/Ero1β specific monoclonal antibodies were re-tested, rat tissue was also prepared in the same way. Figure 5.1A shows a coomassie stained gel of murine tissues matched in protein content to cell tissue lysates. Figure 5.1B shows a Western blot for PDI, which is used here as a loading control. Note that cell lysates give an additional upper background band which is due to cross reactivity with albumin, which is not present in the tissue samples (this was noted in Chapter 3).

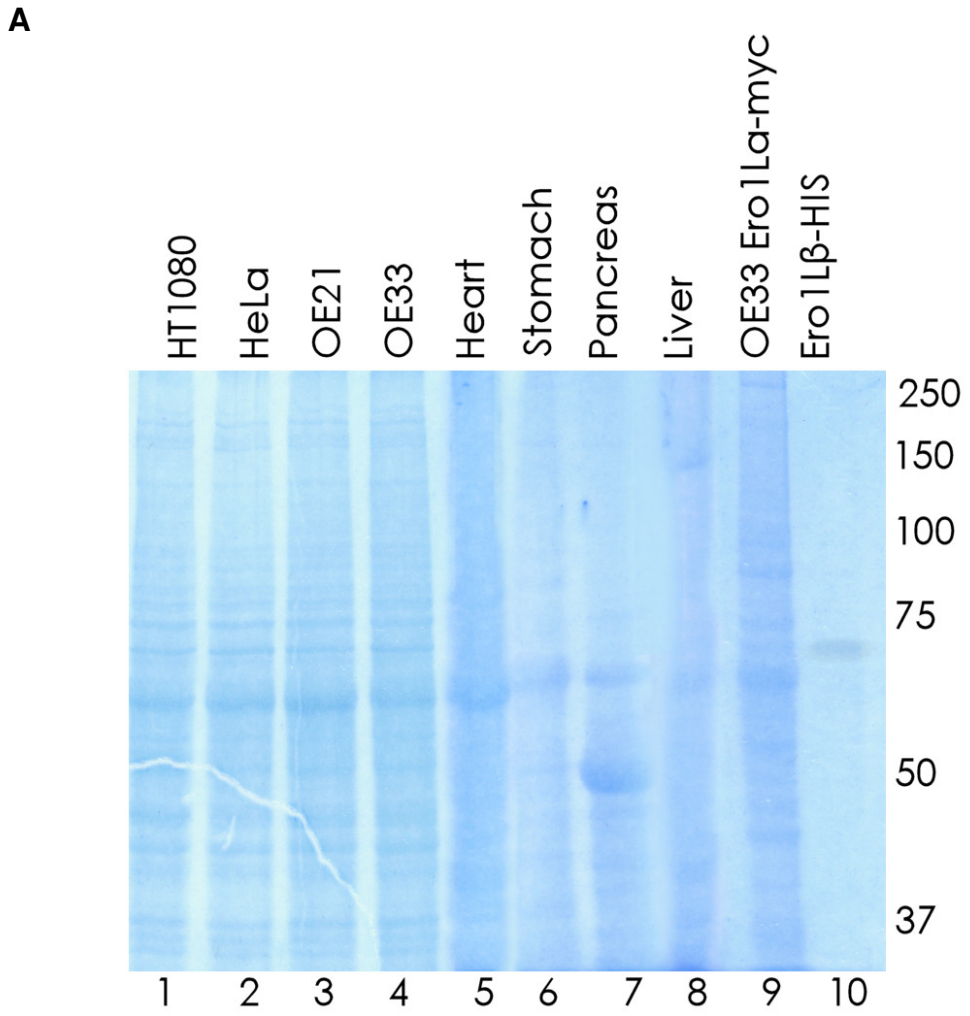

Figure 5.1: Coomassie staining of cell and tissue lysates

Murine tissues and cell lysates were prepared and matched for protein loading, ran using SDS-PAGE and stained with Coomassie blue. A: Matched loading between cell lysates (HT1080, HeLa, OE21 and OE33, lanes 1-4) and murine tissues (Heart, Stomach, Pancreas and Liver, lanes 5-8). An OE33 Ero1α-myc positive transfectant was used as a positive control for Ero1α, and the Ero1β immunogen was used as a positive control for Ero1β (lane 10).

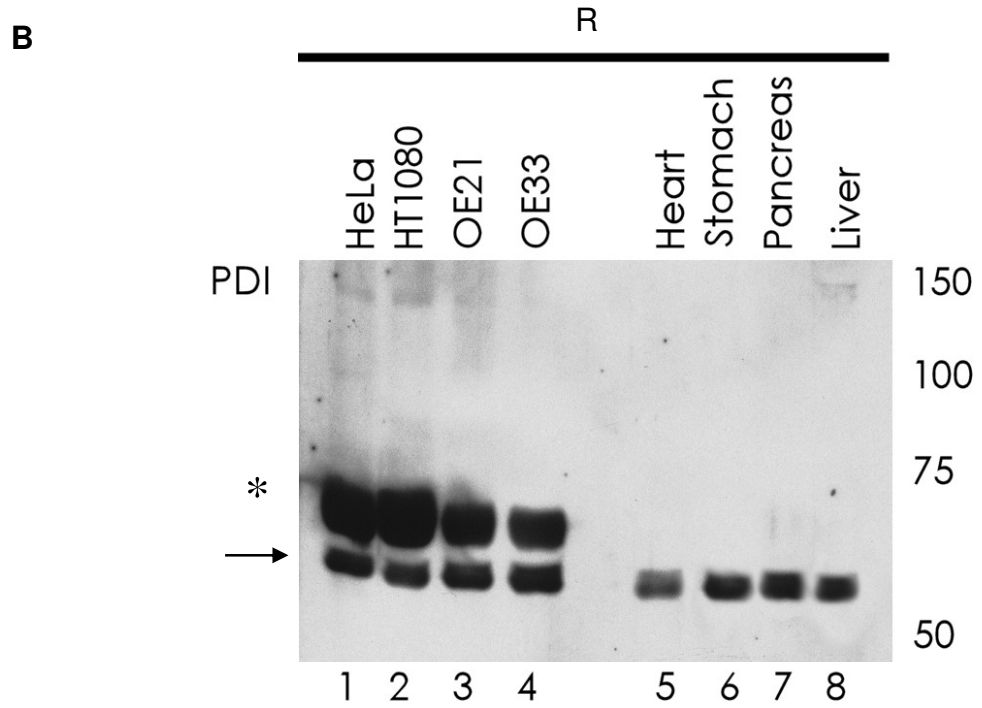

Figure 5.1 (Cont): A demonstration of matched protein loading between cell and tissue lysates

B: Cell and tissue lysates were run on SDS-PAGE and probed by Western blot using the PDI polyclonal antibody. This confirmed equal loading and transfer of protein from the cell and tissue lysates to the membrane. The PDI band is indicated with an arrow. The upper band (*) is a background band, discussed in Chapter 3.

5.2.1.2 Validation of Ero1α and Ero1β positive controls

As the monoclonals being tested were raised in mouse, it was important to show that any positive result on blot was due to specific (anti-mouse) secondary antibodies binding to the primary monoclonal, and that results were not simply due to cross reactivity of the anti-mouse secondary antibody with murine tissue or cell lysates. Thus when the membranes were incubated with secondary antibody alone, no background signals were seen (Figure 5.2).

As the antibody targets were Ero1α and Ero1β, cell culture transfectants positive for myc (Ero1α, Ero1β) or HA (Ero1β) tagged proteins were used in the screening program. These lysates were loaded not only to demonstrate potential protein specificity, but also to demonstrate the presence of the positive control following a re-probe. Figure 5.3 shows blots for Ero1α D5 (Figure 5.3A), anti-myc (Ero1α; Figure 5.3B) and anti-HA (Ero1β; Figure 5.3C). The myc-tagged construct was present in both HeLa and OE33 (Figure 5.3A, lanes 2 and 3), which validated these samples for screening. The HeLa transfectants had two discrete bands, a lower band representing endogenous Ero1α, and an upper band, showing the myc-tagged construct (Figure 5.3A, lane 2). The OE33 transfected sample showed a strong Ero1α-myc tagged band (compare lanes 3 Figure 5.3 A and B), which ran into the weaker endogenous band. The remaining samples (Figure 5.3A, lanes 4-7) show endogenous Ero1α. The anti-myc blot (Figure 5.3B lanes 2-3) confirmed myc expression in Ero1α-myc transfected HeLa and OE33 seen in Figure 5.3A (lanes 2-3). Finally, the expression of Ero1β and the specificity of the HA antibody were also confirmed (Figure 5.3C).

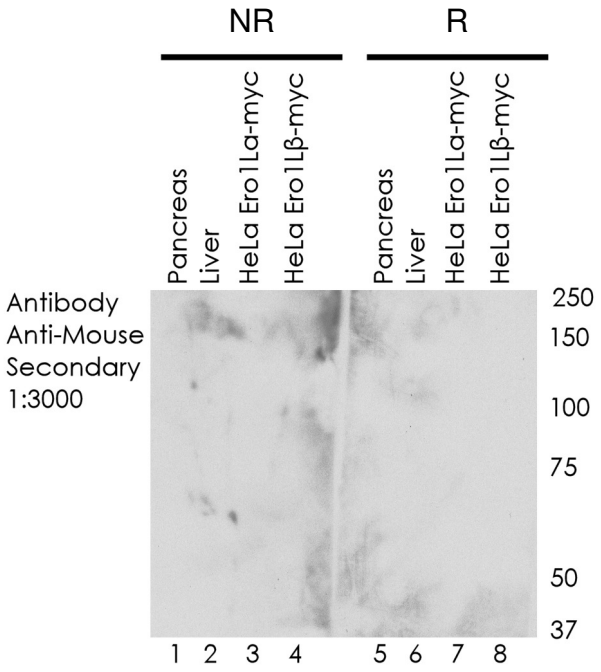

Figure 5.2: No cross-reactivity of the anti-mouse secondary antibody with mouse tissues

Murine pancreas and liver, along with the HeLa Ero1α-myc and Ero1β-myc positive controls, were ran using SDS-PAGE and incubated with the anti-mouse secondary antibody, to verify that no non-specific binding occurs with either the cell or tissue lysates.

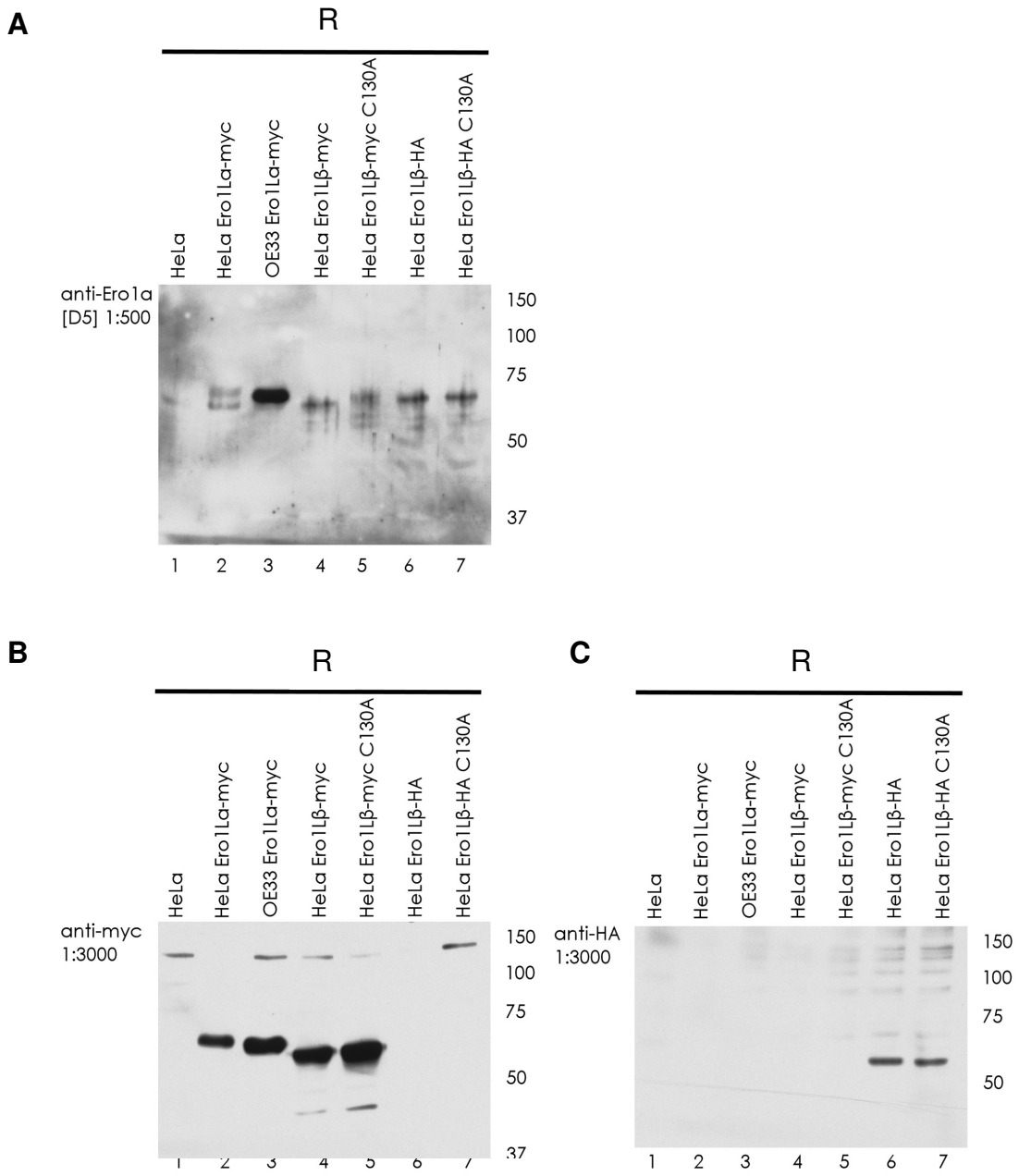

Figure 5.3: Verification of Ero1α and Ero1β positive controls

A: HeLa untransfected and HeLa Ero1α and Ero1β positive controls ran on reducing SDS-PAGE, probed for Ero1α. B: HeLa untransfected and HeLa Ero1α and Ero1β positive controls probed for myc. C: The HeLa negative and positive controls ran using reducing SDS-PAGE and probed for HA.

5.2.1.3 Initial examples of hybridoma supernatants

Initially, both Ero1α and Ero1β derived supernatants were tested against cell and mouse tissue lysates. Ero1α-myc/Ero1β-myc transfected mammalian cells were used as positive controls. Pancreas was used as a positive tissue control for Ero1β. As Ero1α is more widely expressed, heart, pancreas and liver tissue served as positive controls for Ero1α (Dias-Gunasekara *et al.*, 2005).

All samples were run both under non-reducing and reducing conditions. This was to ensure that any potential antibody was tested against different oxidative variants, as an Ero1α/Ero1β redox state-specific monoclonal antibody would be very useful.

Figures 5.4-5.5 show initial test blots using two putative anti-Ero1β supernatants (3.3C4 and 4.5B5), with corresponding anti-myc re-probes to verify the positive controls.

The gels in Figure 5.4 show non-reduced and reduced samples for either cell lysates with the transfected positive controls probed with 3.3C4 (Figure 5.4A, B), or the murine tissue samples, which at this stage included heart, pancreas and liver (Figure 5.4C, D).

Although some bands are seen in Figure 5.4C, notably in the pancreas (Figure 5.4C, lane 2), they were only seen in the tissue lysates, and not the transfectant cell lysates (Figure 5.4A). An anti-myc re-probe confirmed the presence of the positive controls (Figure 5.4B, lanes 5-6, 11-12 and Figure 5.4 D, lanes 5-6, 11-12). Note that under non-reducing conditions, Eros form disulfide bonded higher molecular weight complexes which correspond to Ero-PDI and Ero-ERp44 heterodimers and Ero-Ero homodimers (Anelli *et al.*, 2002, Dias-Gunasekara *et al.*, 2005). Thus it remains possible that these supernatants recognise conformation dependent states of Ero1β.

Figure 5.5 A-D shows data for antibody 4.5B5, a supernatant from Ero1β-HIS immunised mouse hybridoma. This antibody gave blank membranes when tested against cell lysates (Figure 5.5 A,D) but crucially, the anti-myc re-probes showed the presence of the myc-tagged positive controls for Ero1α and Ero1β (Figure 5.5 B,D, lanes 10-12 in the reducing gel).

These preliminary experiments showed promise (Figures 5.4, 5.5 and data not shown) but demonstrated that many of the supernatants were likely to be negative or give non-specific signals, and that all available supernatants would have to be tested systematically to identify an Ero1 specific candidate antibody.

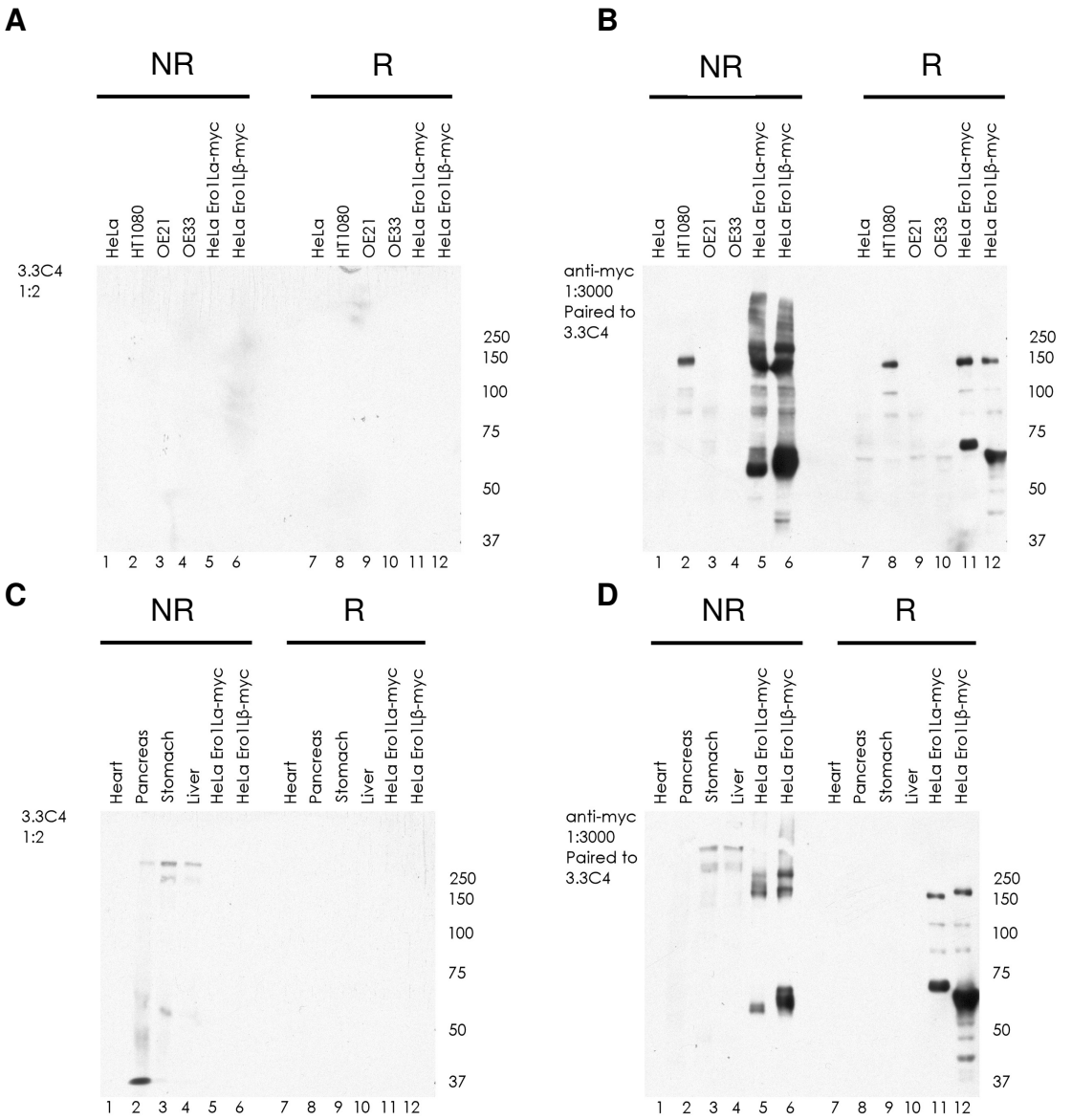

Figure 5.4: Initial test with antibody, 3.3C4 (Ero1β –HIS-GST)

HeLa, HT1080, OE21, OE33 and HeLa Ero1α and Ero1β positive controls ran on reducing SDS-PAGE and probed using A: 3.3C4. B: an anti-myc membrane re-probe, verifying the presence of the positive Ero1α/Ero1β controls. C: Murine heart, pancreas, stomach and liver, along with the HeLa Ero1α/Ero1β positive controls, probed with 3.3C4 and D: an anti-myc re-probe, confirming the presence of the Ero1α-myc/Ero1β-myc controls.

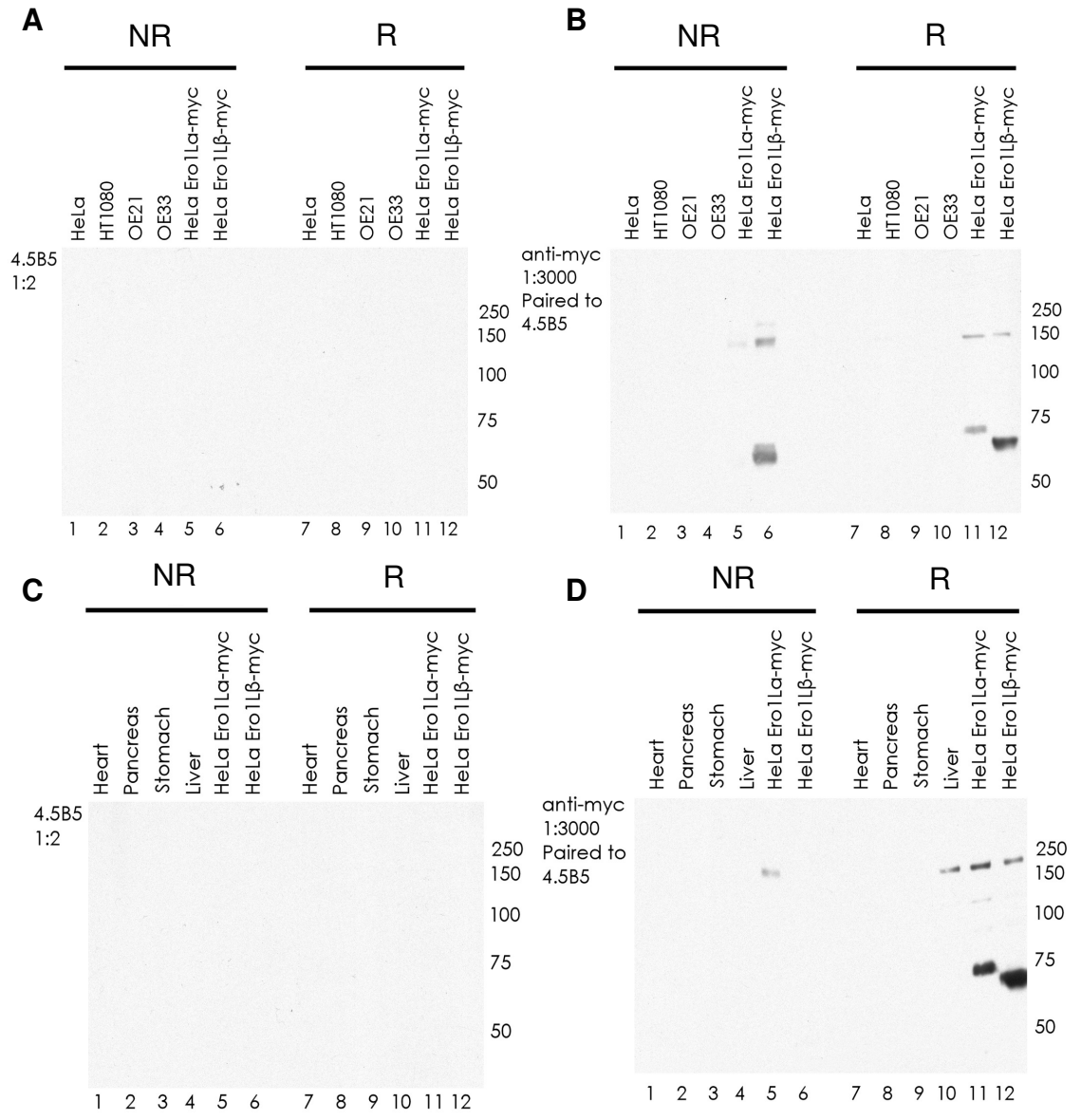

Figure 5.5: Initial test with antibody 4.5B5 (Ero1β-HIS)

A: Western blot probing HeLa, HT1080, OE21 and OE33 cell lysates with 4.5B5. B: An anti-myc re-probe, verifying the presence of the positive Ero1α/Ero1β controls, though the signal in Hela is weak (lane 11). C: Western blot probing murine tissue lysates with 4.5B5. D: An anti-myc re-probe, to verify the presence of the Ero1α-myc/Ero1β-myc controls.

5.2.1.4 Systematic testing of supernatants

Following initial experiments, in the manner described in 5.2.1.3, the following strategy was adopted: murine pancreas and liver tissue was run reducing and non-reducing alongside HeLa Ero1α-myc/Ero1β-myc transfectants. This was done for each hybridoma supernatant. As the same validated myc-positive controls were used (see previous section), anti-myc re-probes are not shown for every blot in this series. The results are shown in Figures 5.6-5.10 below.

Figure 5.6 shows all panels of immunisation Series 1 derived supernatants (Ero1α wildtype). Figure 5.7 and 5.8 shows all panels of immunisation series 2 derived supernatants (Ero1α C397A). Figure 5.9-5.10 shows all panels of immunisation series 3 derived supernatants (Ero1β-HIS-GST). Finally, Figures 5.11-5.14 shows all panels of immunisation series 4 derived supernatants (Ero1β-HIS).

Figure 5.6A shows blot data using antibody 1.2D2, which gave a prominent non-reducing band at 100 kDa, as well as a number of high molecular weight bands in rat pancreas and liver tissue lysate (non-reducing gel, lanes 1-2). As the bands seen were not within the region of 70-50 kDa this antibody was excluded from re-testing. Figure 5.6B shows the remaining Series 1 antibodies that were tested. Each gave a negative result on blot.

Antibody 2.2A3 (Figure 5.7A) showed bands in pancreas and liver tissue lysates between the 100 and 75 kDa markers, and further bands above 100 kDa (lanes 1-2). Though perhaps a little too high to be representative of Ero1α, this was taken for re-testing.

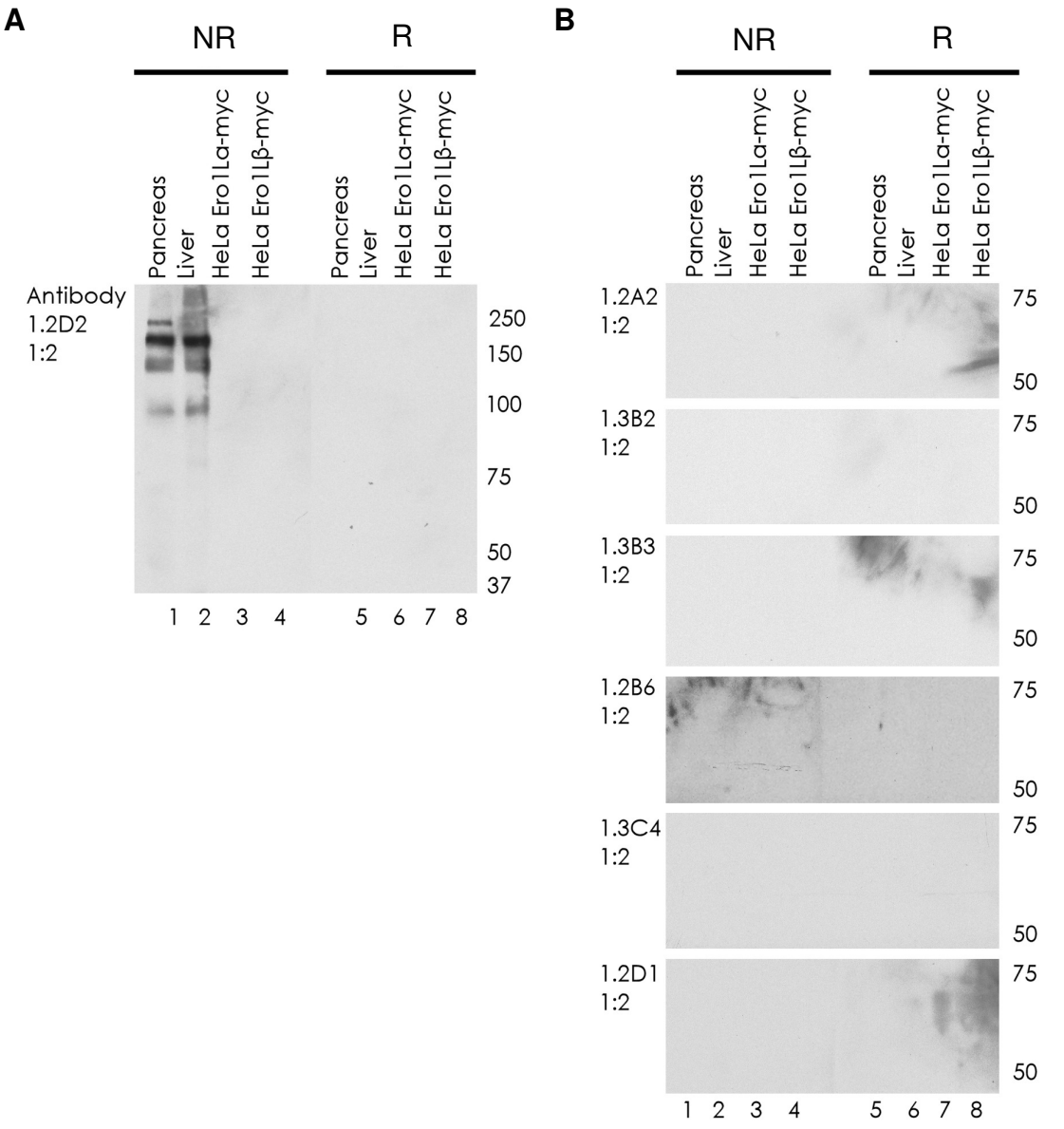

Figure 5.6: Series 1 (Ero1α wildtype) supernatants tested in Western blot

Non-reducing and reducing SDS-PAGE Western blot data showing murine pancreas, liver and HeLa Ero1α/Ero1β transfected positive control lysates probed for A: antibody 1.2D2; this antibody was not earmarked for re-testing. B: None of the supernatants listed in this panel showed any specificity to the immunogen, Ero1α (wildtype); the blot regions have been restricted to the target area of between 75 and 50 kDa. These blots were re-probed to verify that the samples were capable of giving a signal (to PDI, not shown, but exemplified earlier in the chapter).

2.2C4 (Figure 5.7B) showed bands between the target area of 75 and 50 kDa in the HeLa transfectants in the non-reducing, but not the reducing gel (lanes 3-4), and was selected for re-testing. 2.2C6 (Figure 5.7C) also showed bands in the 75-50 kDa region in non-reduced pancreatic and liver tissue (lanes 1-2). 2.3B3 (Figure 5.7D) showed diffuse smearing in pancreatic and liver lysates, but with some specific bands appearing above 100 kDa (lanes 1-2). Finally in this series, 2.3C4 (Figure 5.8A) showed smearing in non-reduced pancreas and liver tissue (lanes 1-2), but there was a band in each of these samples within the 75-50 kDa region that warranted further testing. Negative data generated with the remaining supernatants in Series 2 is shown in Figure 5.8B.

Figure 5.9 shows data from Series 3 (Ero1β-HIS-GST) antibodies, 3.3A6 and 3.3C2 which both show high molecular weight bands in pancreatic and liver tissue (non-reducing gel, lanes 1-2, each blot). Of note, 3.3A6 gave two bands around 50 kDa (lanes 1-2), and 3.3C2 showed a prominent band at 75 kDa in pancreas only (lane 1), the Ero1β-expressing tissue. The remaining (negative) antibodies in the series are shown in Figure 5.10A-B.

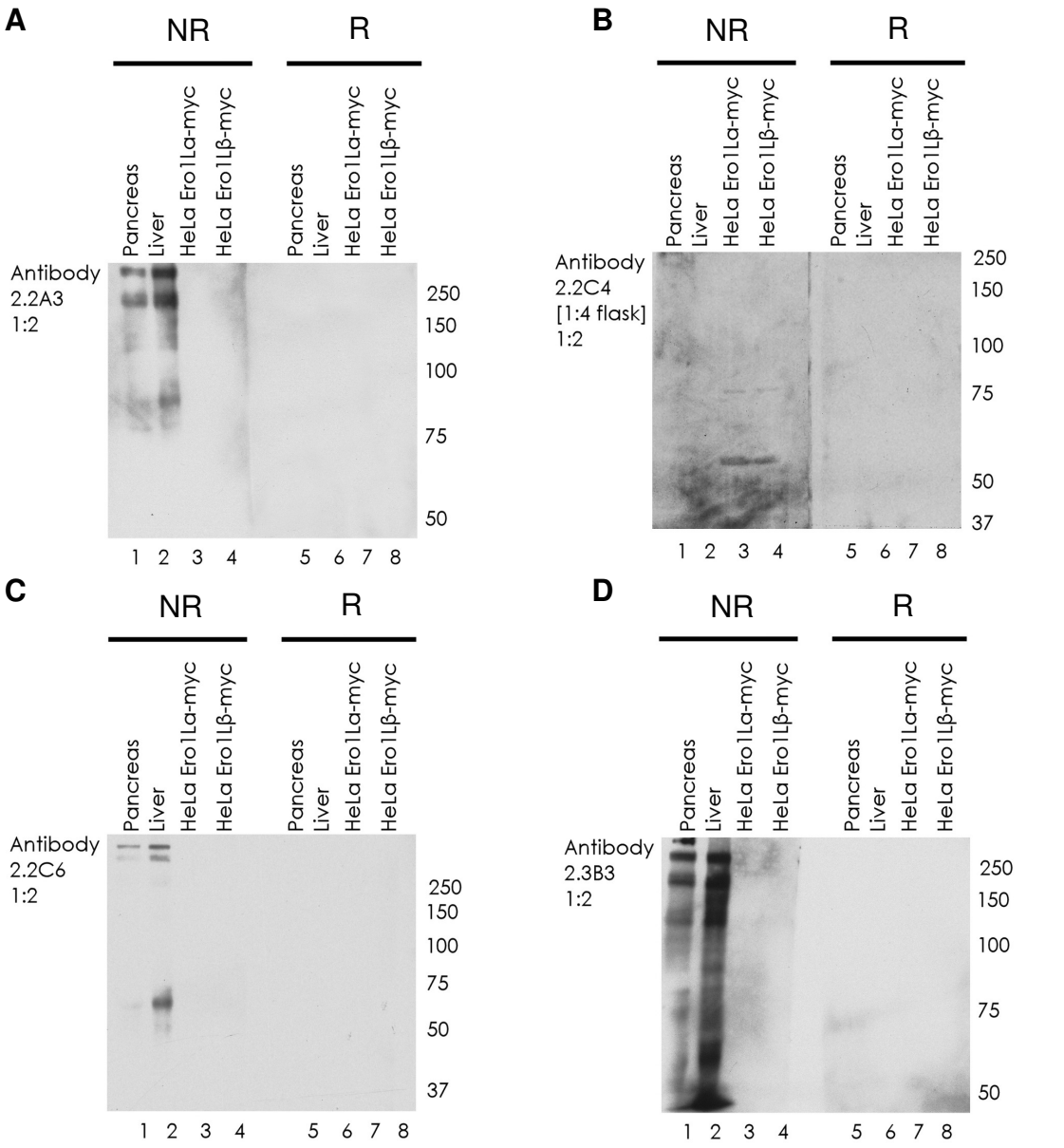

Figure 5.7: Series 2 (Ero1α C397A) supernatants tested in Western blot

Non-reducing and reducing SDS-PAGE Western blot data showing murine pancreas, liver and HeLa Ero1α/Ero1β transfected positive control lysates analysed by SDS-PAGE and probed using series 2 antibodies; A: 2.2A3, B: 2.2C4, C: 2.2C6 and D: 2.3B3. 2.2A3, 2.2C4 and 2.2C6 were earmarked for re-testing.

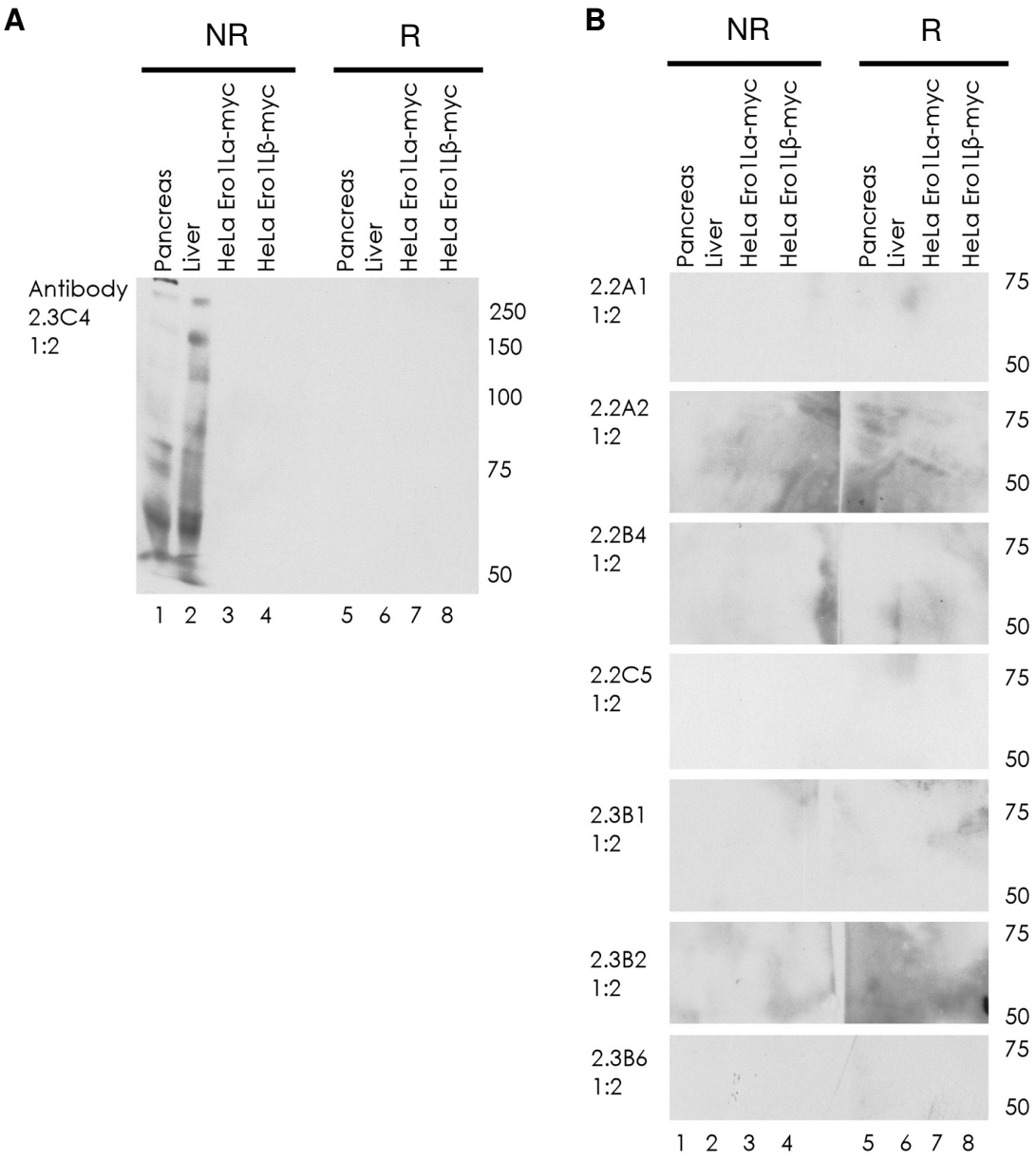

Figure 5.8: Series 2 (Ero1α C397A) supernatants tested in Western blot

Non-reducing and reducing SDS-PAGE Western blot data showing murine pancreas, liver and HeLa Ero1α/Ero1β transfected positive control lysates probed for A: 2.3C4. B: Clipped panel blots in the 75 and 50 kDa region, for antibodies 2.2A1, 2.2A2, 2.2B4, 2.2C5, 2.3B1, 2.3B2, and 2.3B6. No antibodies from A or B were taken for re-testing. These blots were re-probed for PDI to verify protein transfer (not shown).

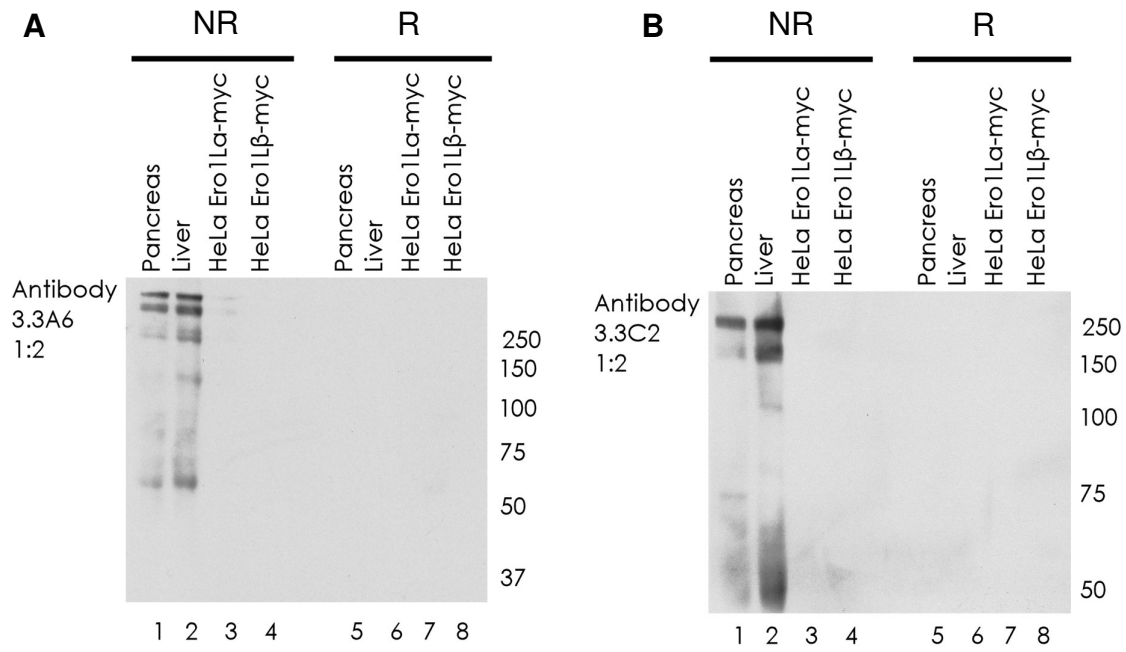

Figure 5.9: Series 3 (Ero1β-HIS-GST) supernatants tested in Western blot

Non-reducing and reducing SDS-PAGE Western blot data showing murine pancreas, liver and HeLa Ero1α/Ero1β transfected positive control lysates probed for A: 3.3A6 and B: 3.3C2. Both of these antibodies were taken for re-testing. These were re-probed for PDI to verify transfer (not shown).

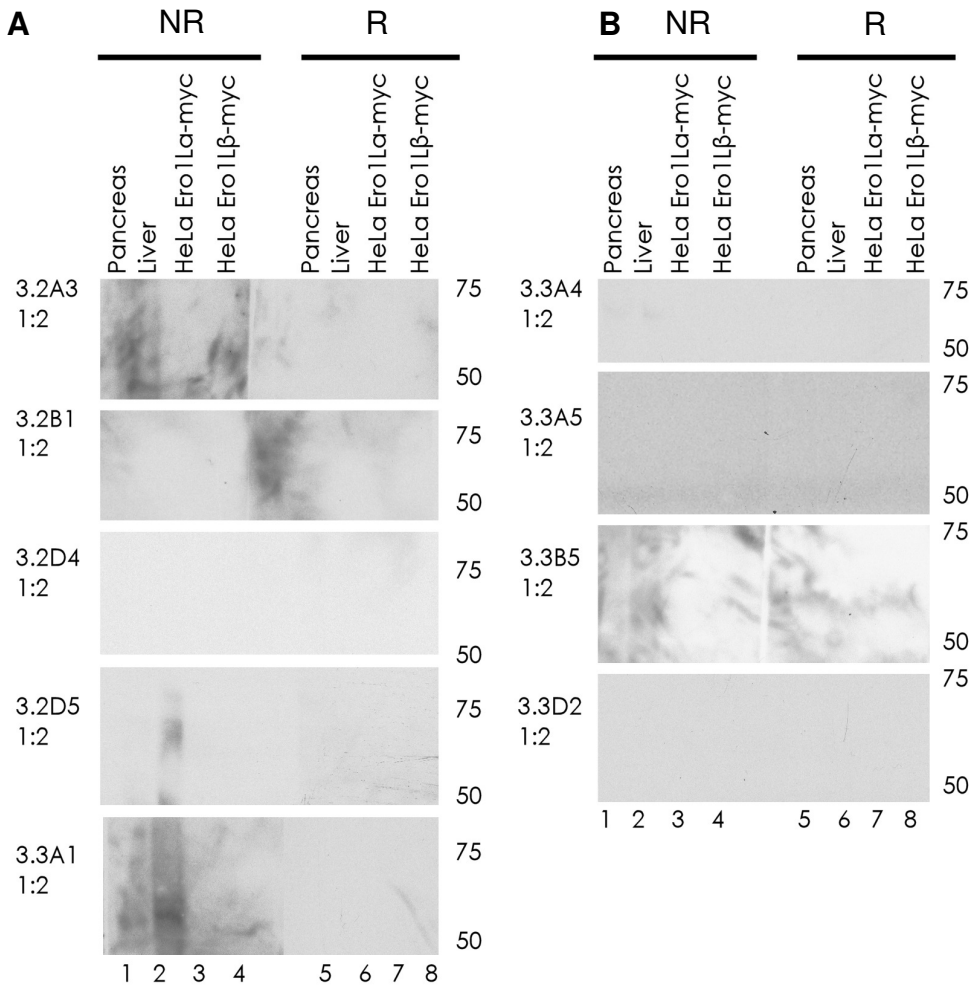

Figure 5.10: Series 3 (Ero1β-HIS-GST) supernatants tested in Western blot

These clipped panel blots in the 75 and 50 kDa region show Western blot data for each of the remaining antibodies in the series. A: 3.2A3, 3.2B1, 3.2D4, 3.2D5, 3.3A1 and B: 3.3A4, 3.3A5, 3.3B5 and 3.3D2. These did not show signs of Ero1β specificity. Both 3.2D5 and 3.3A1 produced a mild smear in non-reduced liver tissue lysate, though this was judged to be too non-specific, and there was no signal seen in the pancreas lysate. These were re-probed for PDI to verify transfer (not shown).

Figure 5.11 shows Western blot data from the final set of supernatants, Series 4 (Ero1β-GST) which led to eight antibodies from this series being re-tested. Antibody 4.3A4 (Figure 5.11A) showed 75 kDa and~45 kDa bands in the liver lysate sample on the reducing gel only (lane 6). The specific band around the 75 kDa region made it suitable for a re-test. Antibody 4.3B1 (Figure 5.11B) showed specific bands in the >100 kDa region in pancreas and liver tissue lysate in the non-reducing gel (lanes 1-2), but not in the reducing gel. Along with 4.4B2 (Figure 5.11C), which showed bands >150 kDa (lanes 5-6) and 4.4C6 (Figure 5.11D) which showed a single ~150 kDa band (lane 1), they were candidates for the re-testing phase of candidate Ero1β specific and pan-Ero specific monoclonals. 4.4D4 (Figure 5.12A) showed a number of bands in pancreas (lane 5), and a smear in the reduced liver tissue lysate (lane 6), with bands in both samples at the upper and lower ends of the 75-50 kDa region. Antibody 4.5B3 (Figure 5.12B) showed a specific band at around 90 kDa in the liver lysate, which should be negative for Ero1β expression (Figure 5.12B lane 2) on the non-reducing gel, and higher molecular weight bands also seen in non-reduced pancreas lysate (lane 1). This was taken also for re-testing. 4.6A1 (Figure 5.12C) showed a number of high molecular weight bands against non-reduced pancreas (lane 2) and liver lysate (lane 3), but not heart lysate (lane 1). Note the two principle bands around 50 kDa in pancreas and liver lysate (lanes 2-3). 4.6C2 (Figure 5.12D) showed a potentially interesting band around the 50 kDa region, in reduced liver tissue only. Figure 5.13 A-B and Figure 5.14 shows the remaining supernatants used in Western blot experiments that were not chosen for re-testing, having produced a negative result on blot. This was taken as a potential Ero1β negative monoclonal for the re-testing process.

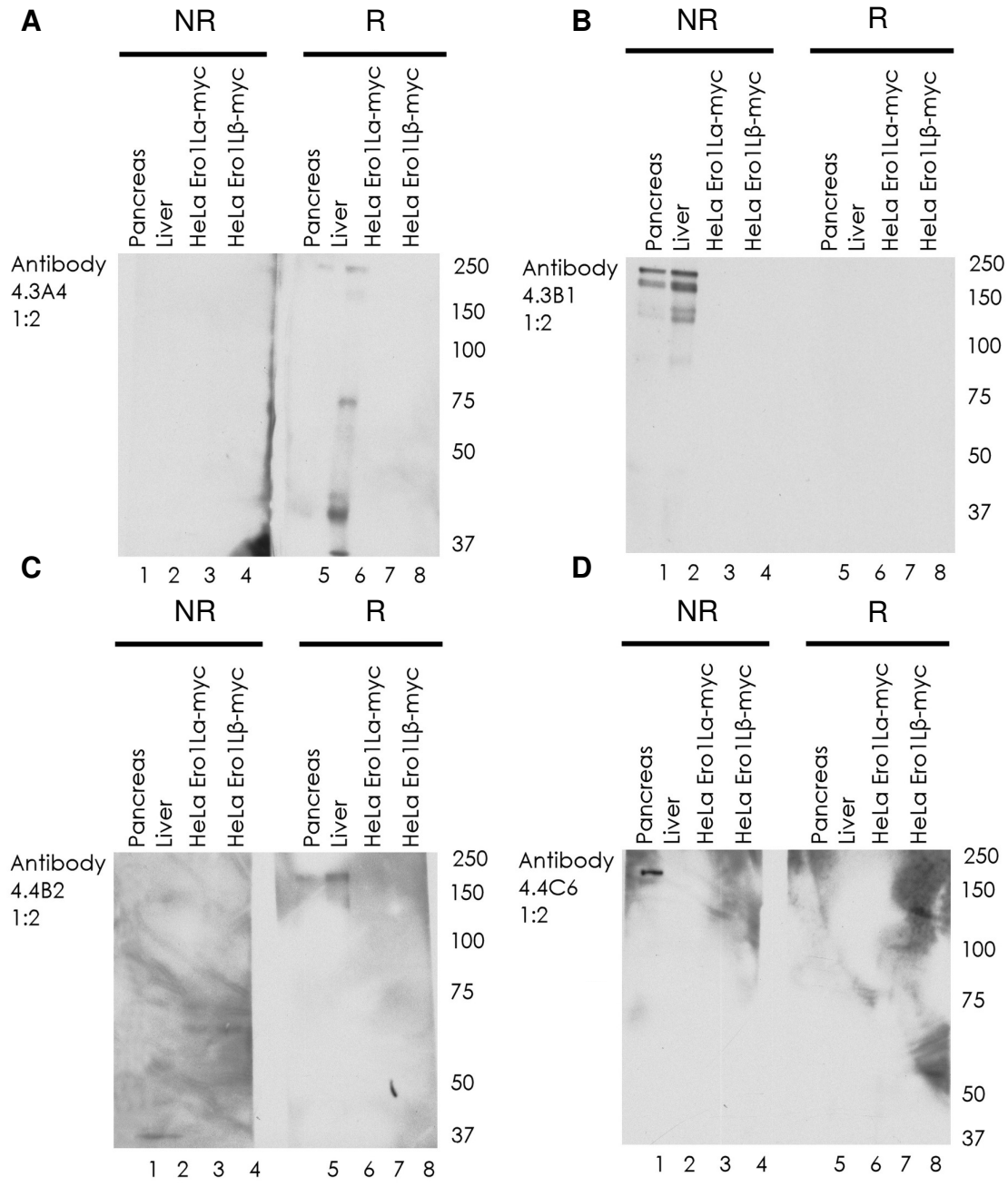

Figure 5.11: Series 4 (Ero1β-HIS) supernatants tested in Western blot

Non-reducing and reducing SDS-PAGE Western blot data showing murine pancreas, liver and HeLa Ero1α/Ero1β transfected positive control lysates probed for A: 4.3A4; B: 4.3B1; C: 4.4B2 and D: 4.4C6. Antibodies 4.3A4, 4.3B1 and 4.4C6 were taken for re-testing. These membranes were re-probed for PDI to verify transfer (not shown).

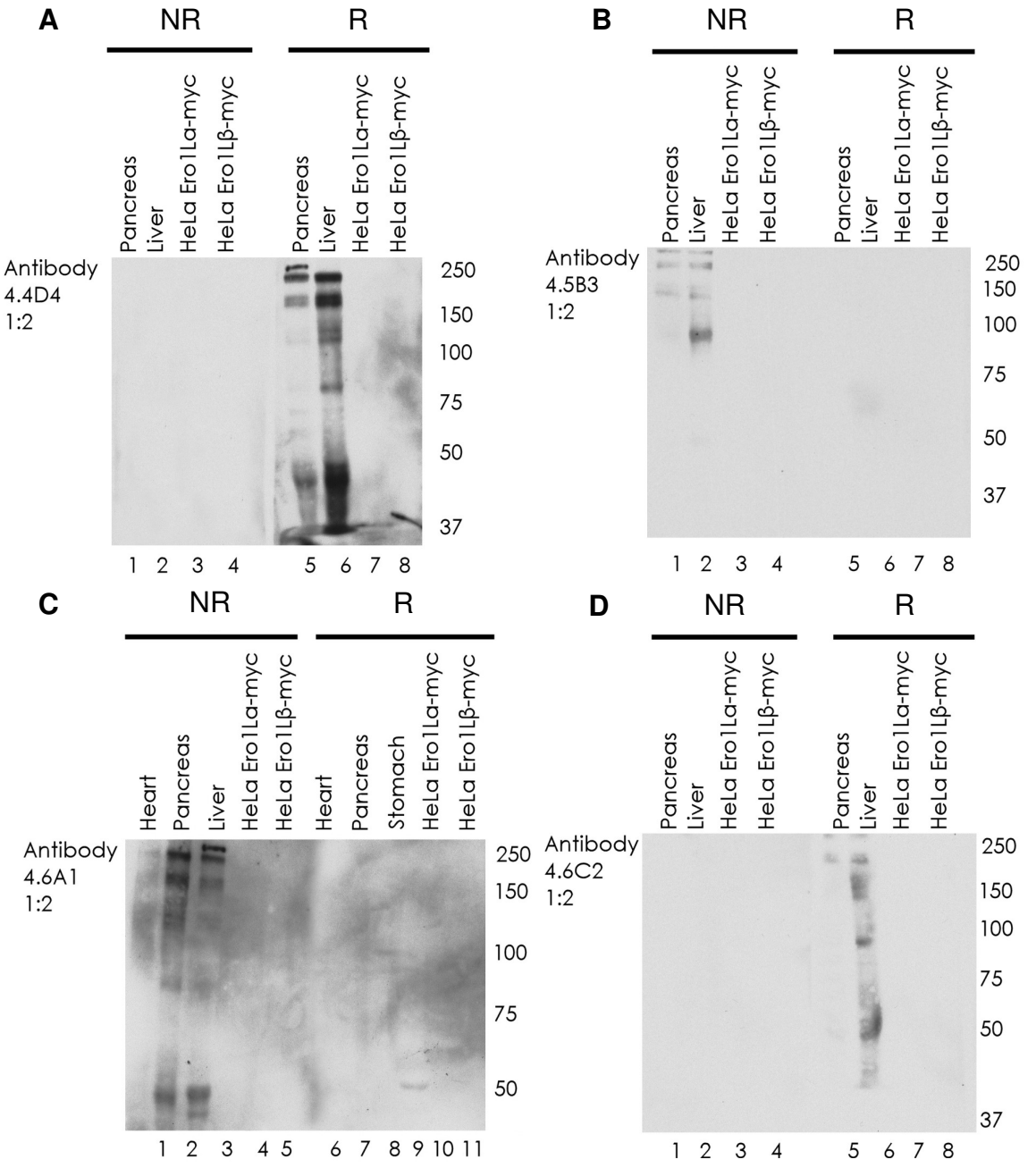

Figure 5.12: Series 4 (Ero1β-HIS) supernatants tested in Western blot

Non-reducing and reducing SDS-PAGE Western blot data showing murine pancreas, liver and HeLa Ero1α/Ero1β transfected positive control lysates probed for A: 4.4D4; B: 4.5B3; C: 4.6A1; D: 4.6C2. Each of these antibodies was taken for re-testing. These membranes were re-probed for PDI to verify transfer (not shown).

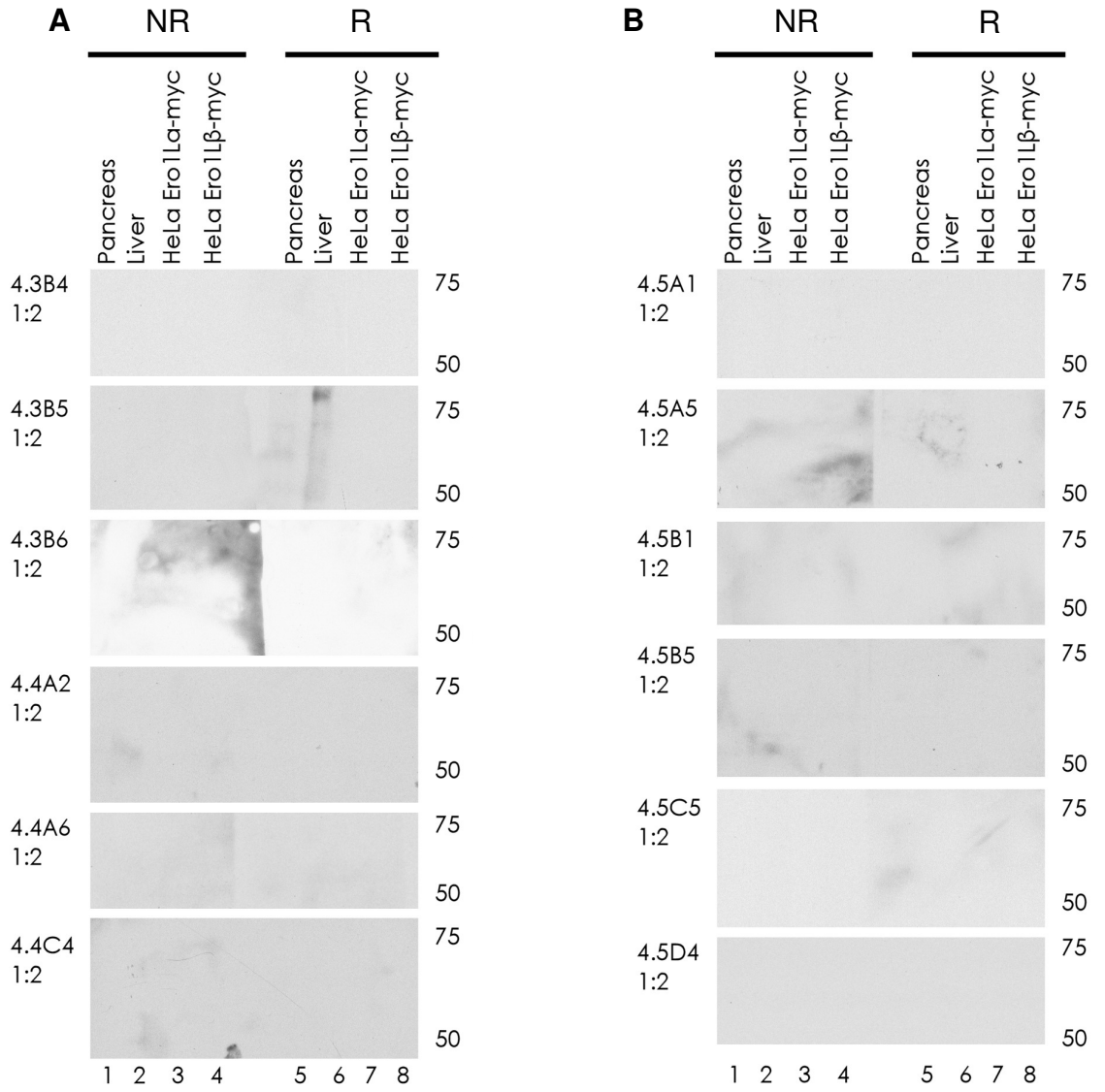

Figure 5.13: Series 4 (Ero1β-HIS) supernatants tested in Western blot

A and B: These clipped panel blots in the 75 and 50 kDa region show Western blot data for each of the remaining antibodies in the series. Each antibody used is labelled next to the blot panels. None of these supernatants were selected for re-testing. These were re-probed for PDI to verify transfer (not shown).

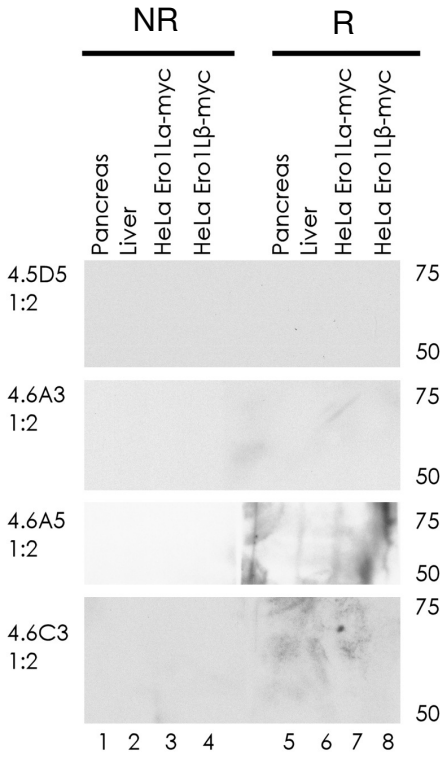

Figure 5.14: Series 4 (Ero1β-HIS) supernatants tested in Western blot

This panel continues to show negative data for the 75-0 kDa region for each of the remaining series 4 antibodies in the series. Each antibody used is labelled next to the blot panels. These were re-probed for PDI to verify transfer (not shown).

5.2.1.5 Re-testing of potentially positive monoclonals

The supernatants for each immunogen series that were identified as potentially giving a specific signal were re-tested (these were discussed in turn in the preceding figures 5.4-5.14). Changes to the screening strategy were the inclusion of rat pancreas and liver tissue, the use of OE33 cell lysates and for the Ero1β series, the addition of a 10μg sample of Ero1β immunogen that was used to immunise the animals.

Figure 5.15 shows all panels of immunisation series 2 derived supernatants (Ero1α C397A). Figure 5.16 shows all panels of immunisation series 3 derived supernatants (Ero1β-HIS-GST). Figure 5.17 and 5.18 shows all panels of immunisation series 4 derived supernatants (Ero1β-HIS).

In the Series 2 re-test, 2.2A3 (Figure 5.15A), there was a strong band present in murine rat liver, around 250 kDa (lane 3). In contrast to the original blot (see (Figure 5.7A) there was no signal seen in mouse pancreas tissue (lanes 1-2). Two alternative 2.2C4 supernatants were available for testing, from two different hybridomas at different passage numbers. With 2.2C4 (I) (Figure 5.15B), there was a band seen just above 50 KDa, which could correspond to Ero1α in OE33 (lane 10). This was also seen in the HeLa transfected samples in Figure 5.7B. As Ero1α is highly expressed in OE33 (see Chapter 3) this is still a potential candidate antibody for Ero1α, although it did not show a signal with either rat or murine tissue, indicating possible non-specificity to rat/murine Ero1α. Antibody 2.2C4 (II) (Figure 5.15C) is an alternate preparation of 2.2C4, and showed a different result than 2.2C4 (I). Here, there were high molecular weight bands seen in mouse/rat pancreas and liver (lanes 1-4), and ~80kDa bands in rat pancreas and rat/mouse liver (lanes 2-4). There were also bands present <50kDa (lanes 1, 3, 4). This was not consistent with what would be expected of an Ero-specific antibody, and neither was there a signal seen in reduced OE33 (lane 10). 2.2C6 (Figure

5.15D) shows a large band present in mouse liver, likely an aggregate of high molecular weight proteins.

In the re-tests of 3.3A6 (Figure 5.16A), a band around 50 kDa in rat liver was seen (lane 4). Additional high molecular weight bands were present in the non-reduced tissue sample of rat pancreas (lanes 2). In contrast to the original test of 3.3A6, (seen in Figure 5.9A), the weakly-seen murine pancreatic band was not present here, nor was there a sufficient signal in OE33 to suggest 3.3A6's specificity to Ero1α. The re-test of 3.3C2 (Figure 5.16B) was broadly reproduced from the original test (seen in Figure 5.9), but showed no specificity to the Ero1β immunogen.

Figures 5.17 and 5.18 show the re-test data of candidate Ero1β-HIS monoclonals. Though both 4.3A4 (Figure 5.17A) and 4.3B1 (Figure 5.17B) were chosen for re-testing, no band was seen in the immunogen sample, lane 6, and as such, these were unlikely to be specific to Ero1β, despite showing a diffuse number of bands in mouse/rat tissues (lanes 1-4). 4.4C6 (Figure 5.17C) shows a number of high molecular weight bands in each of rat pancreas, and rat liver. A band between 50 and 37kDa in rat liver (lane 5) was interesting, though unlikely to be Ero1β as liver does not express Ero1β under non-stress conditions. An absence of a signal in the Ero1β immunogen lane also suggested that 4.4C6 was not Ero1β-specific. In the re-tests of 4.4D4 (Figure 5.17D) and 4.5B3 (Figure 5.18A) there were some specific bands. However, the absence of a signal against the fusion protein suggested neither of these two were specific for Ero1β. Antibody 4.4D4 showed similar high molecular weight bands in both murine and rat liver tissue (lanes 3-4), though these were unlikely to be Ero1β. 4.5B3 showed similar high molecular weight bands in both rat pancreas and liver, though these are also unlikely to be Ero1β as liver is Ero1β-negative (lanes 2, 4).

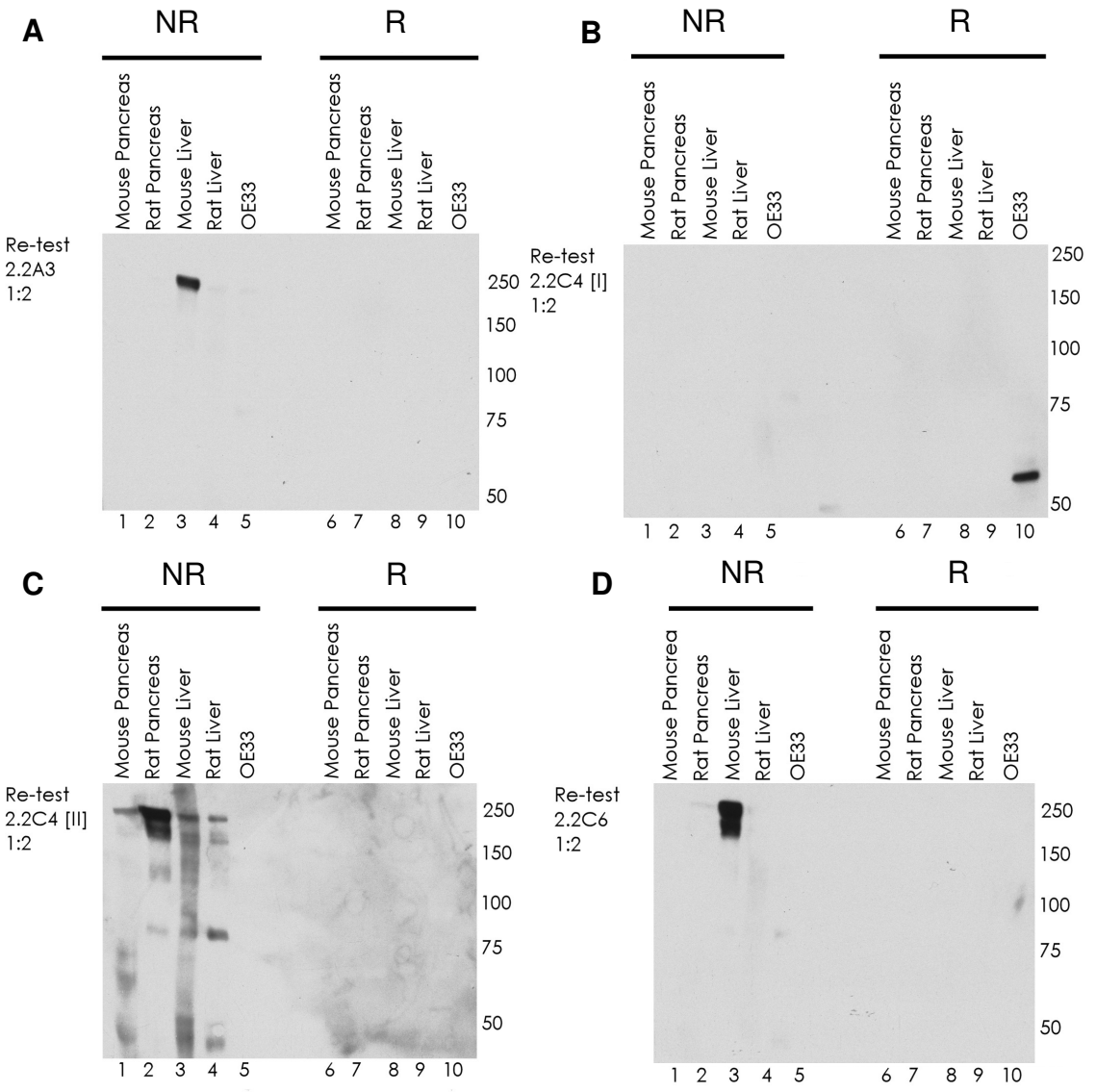

Figure 5.15: Retest of Series 2 (Ero1α C397A)

Non-reducing and reducing SDS-PAGE Western blot data showing murine and rat pancreas, liver lysates and OE33 Ero1α positive control lysate probed for A: 2.2A3, B: 2.2C4 (I), C: 2.2C4 (II) D: 2.2C6.

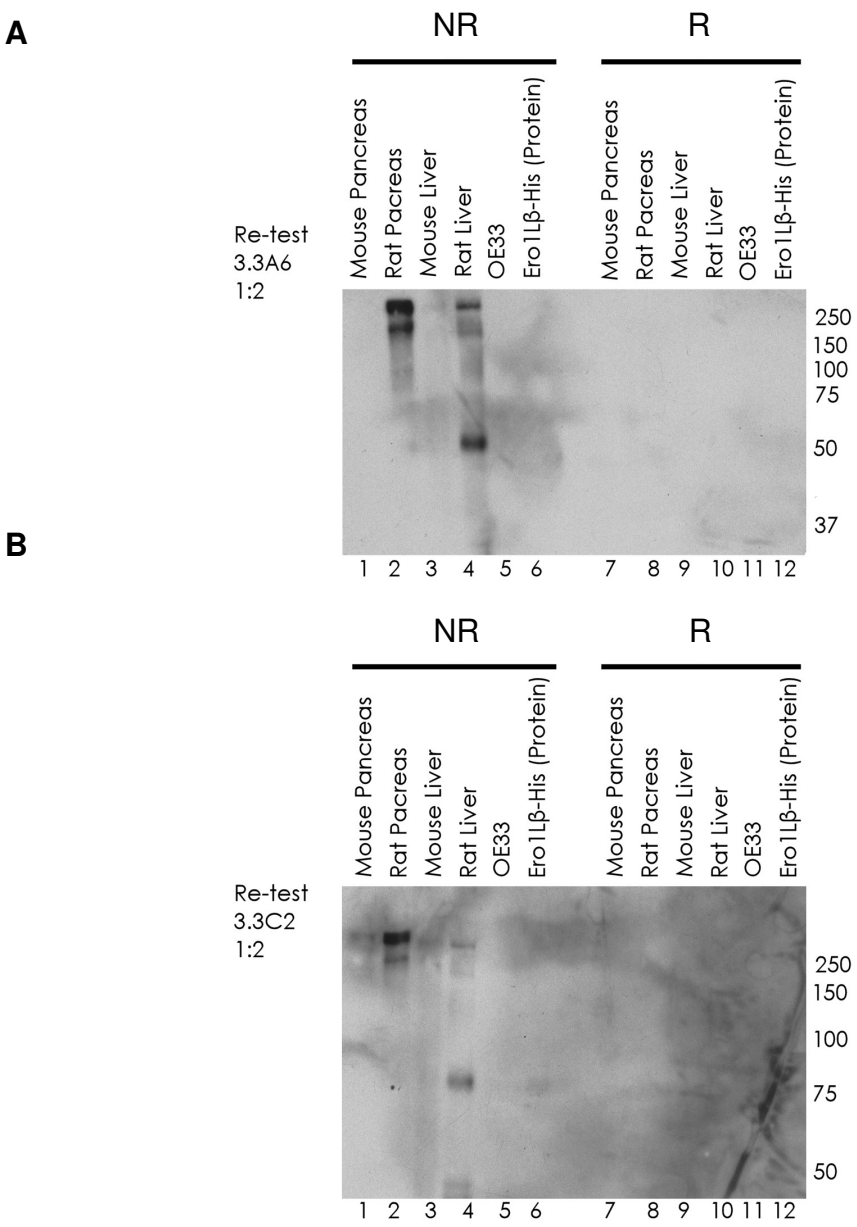

Figure 5.16: Re-test of Series 3 (Ero1β-HIS-GST)

Non-reducing and reducing SDS-PAGE Western blot data showing murine and rat pancreas, liver lysates, OE33 Ero1α positive control and Ero1β immunogen positive control probed for Series 3 Ero1β candidate monoclonals A: 3.3A6 and B: 3.2C2.

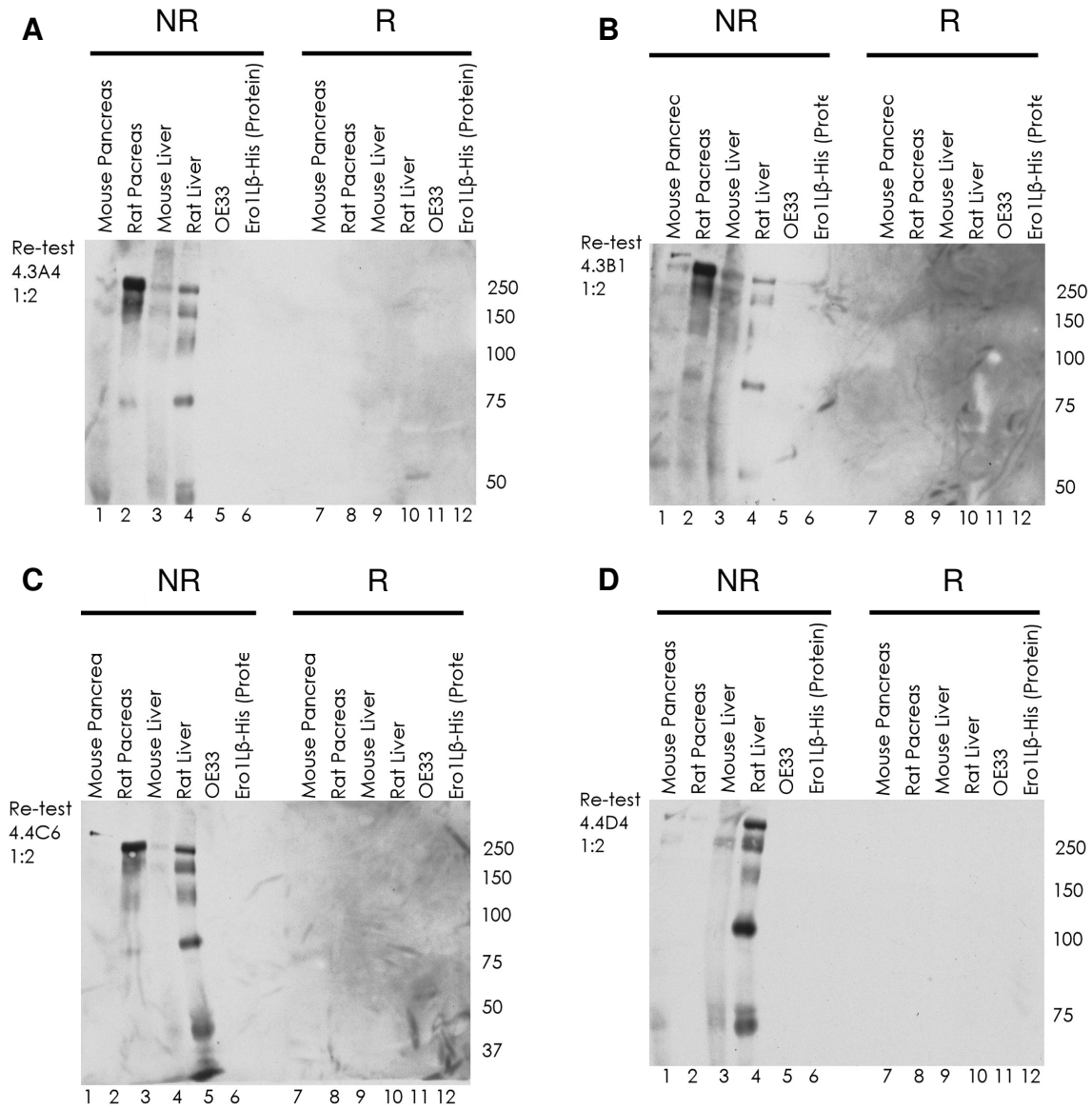

Figure 5.17: Re-test of Series 4 (Ero1β-HIS)

Non-reducing and reducing SDS-PAGE Western blot data showing murine and rat pancreas, liver lysates, OE33 Ero1α positive control and Ero1β immunogen positive control probed for Series 3 Ero1β candidate monoclonals A: 4.3A4; B: 4.3B1; C: 4.4C6, and. D: 4.4D4.

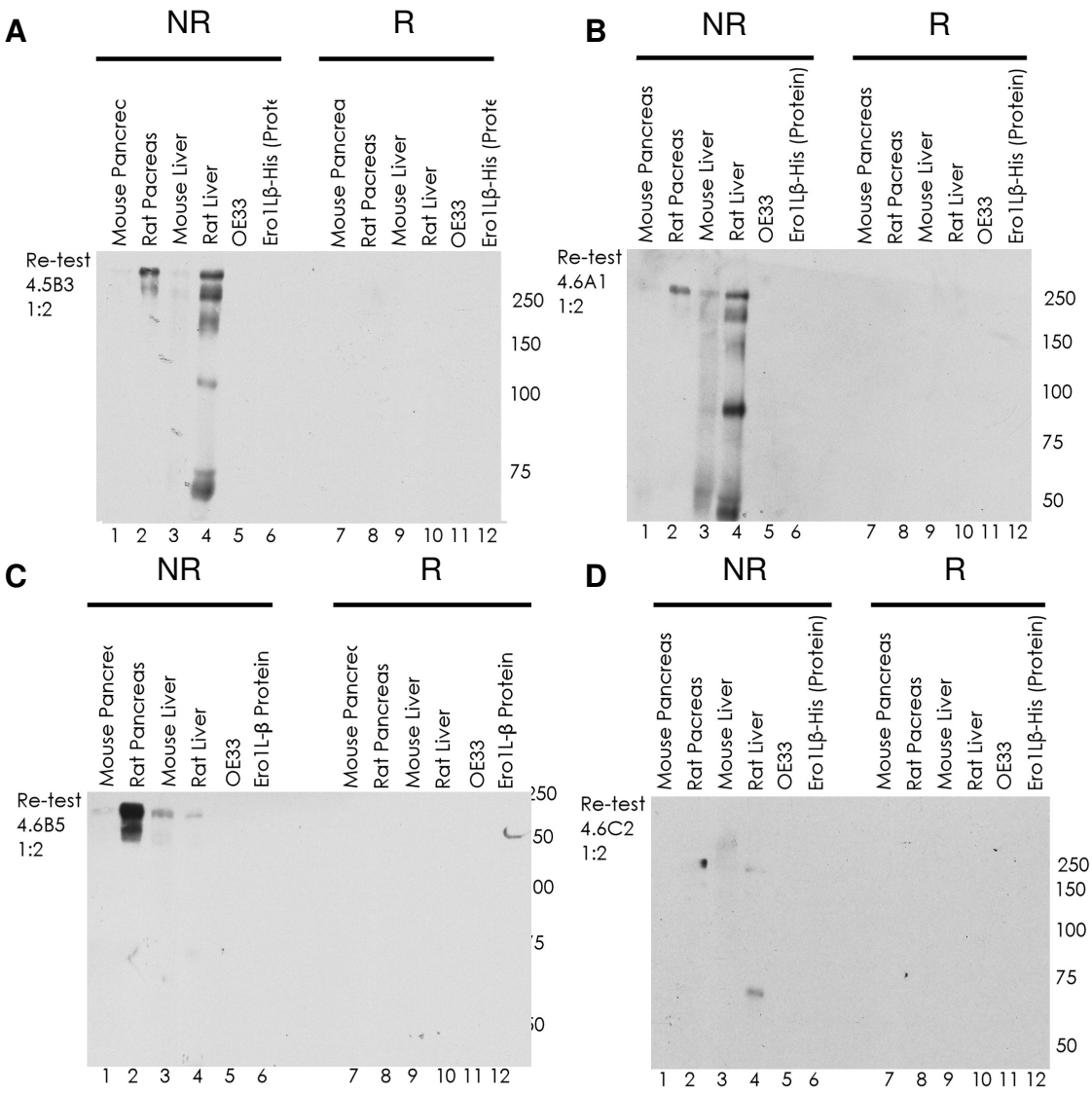

Figure 5.18: Re-test of Series 4 (Ero1β-HIS)

Non-reducing and reducing SDS-PAGE Western blot data showing murine and rat pancreas, liver lysates, OE33 Ero1α positive control and Ero1β immunogen positive control probed for Series 3 Ero1β candidate monoclonals A: 4.5B3; B: 4.6A1; C: 4.6B5 and D: 4.6C2.

The re-test of 4.6A1 showed a similar result in rat tissue here as it did with mouse tissue in initial testing (Figure 5.12C), though no signal with the Ero1β fusion protein was seen (Figure 5.18B, lane 12). 4.6B5 (Figure 5.18C) showed some very high molecular weight bands, though once again nothing with the fusion protein at the correct molecular weight (lane 12). As such neither of these was likely to be specific to Ero1β. This re-test of 4.6C2 was also similar to its initial test (Figure 5.12D) with a weak signal for rat liver, (Figure 5.18D, lane 6) but not mouse, in the region where one would expect an Ero signal. However, there was no signal seen with the fusion protein (lane 12) or in liver (lane 3-4). 4.6C2 is unlikely to recognise Ero1α (a possibility if the epitope is similar between Eros) as there was no signal seen against the OE33 sample (lane 11).

Following re-testing, it was clear that a number of supernatants recognised non-reduced, high molecular weight bands in either pancreas or liver. Further analysis is required to see whether these antibodies recognised Eros in higher molecular weight complexes, and whether they are conformation specific.

5.2.1.6 Re-testing 2.2C4 in cell immunofluorescence

The 2.2C4 antibody, raised against mutant Ero1α (C397A), was further tested in cell immunofluorescence experiments. Supernatant 2.2C4 showed an Ero1α-like signal at the correct molecular weight, in some cells only, and higher molecular mass complexes in tissue (see sections 5.2.1.4-5.2.1.5).

As our current lab antibodies for Ero1α are not conformation specific, a positive outcome here with 2.2C4 would be beneficial. The cell lines tested were HeLa and OE21. Ero1α expression in HeLa is sufficient to detect Ero1α on blot, whilst OE21 made a suitable comparison for a cell line which expresses low levels of Ero1α. OE33 cells were not used because of their tendency to clump. Figure 5.19 shows

immunofluorescence data for ER tracker in HeLa (Figure 5.19A) and OE21 (Figure 5.19B). Similarly, Figure 5.20 shows immunofluorescence data for PDI in HeLa (Figure 5.20A) and OE21 (Figure 5.20B). From these figures, the characteristic ER staining was seen (Figure 5.19) and it is clear that both HeLa and OE21 express PDI, which localised to the ER around the DAPI stained nuclei.

Figure 5.21A shows immunofluorescence data where 2.2C4 has been used to stain HeLa and OE21. This is in comparison to Figure 5.16B, which shows immunofluorescence data for 2.3C4; a supernatant which had not been identified as a candidate for Ero1α specificity. Neither 2.2C4 nor 2.3C4 showed ER localisation, when compared to Figure 5.19 (ER tracker) or Figure 5.20 (PDI).

Although 2.2C4 gave a dull fluorescence in HeLa, this was not seen in OE21. The results suggest that 2.2C4 is not suitable for use in immunofluorescence.

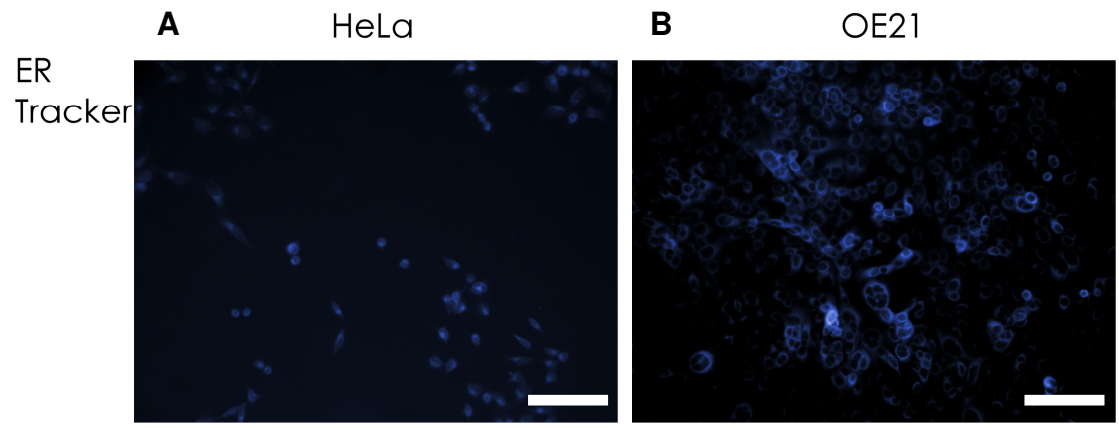

Figure 5.19: Immunofluorescence signal of ER tracker in HeLa and OE21

A: Untreated HeLa and B: OE21 cells were fixed and permeabilised and stained using ER tracker. The blue staining of the ER tracker leaves a punctuate, unstained spot in the centre, indicating the cell nucleus. Scale bars 100 µm.

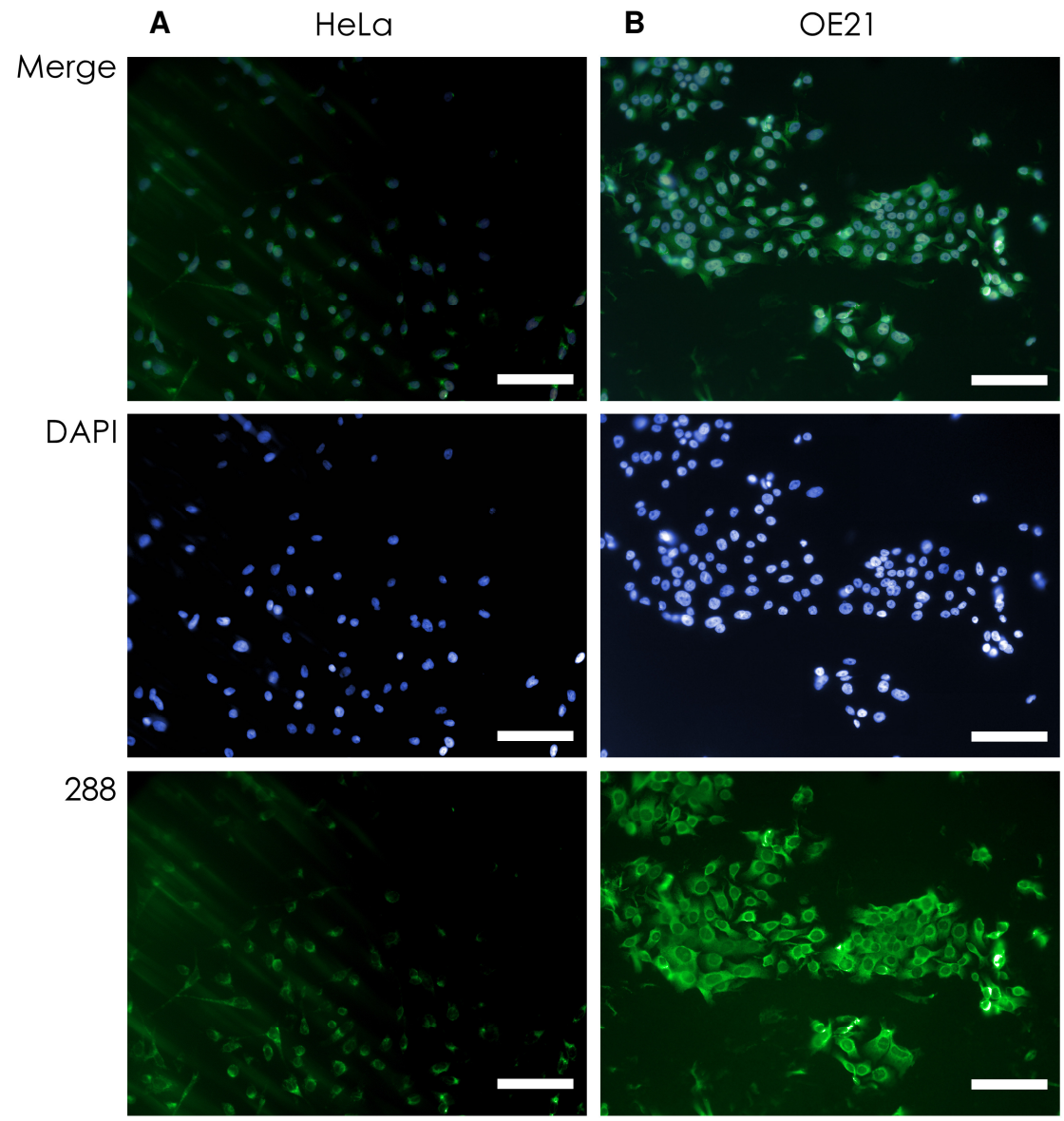

Figure 5.20: Immunofluorescence signal of PDI in HeLa and OE21

A: Untreated HeLa and B: OE21 cells were fixed and permeabilised and probed for PDI. DAPI panels showed blue nuclear staining. The "Merge" panel shows green PDI staining overlaid onto blue DAPI staining, indicating cell nuclei. The perinuclear localisation of PDI here is consistent with the perinuclear location of the ER. Scale bars 100 μm.

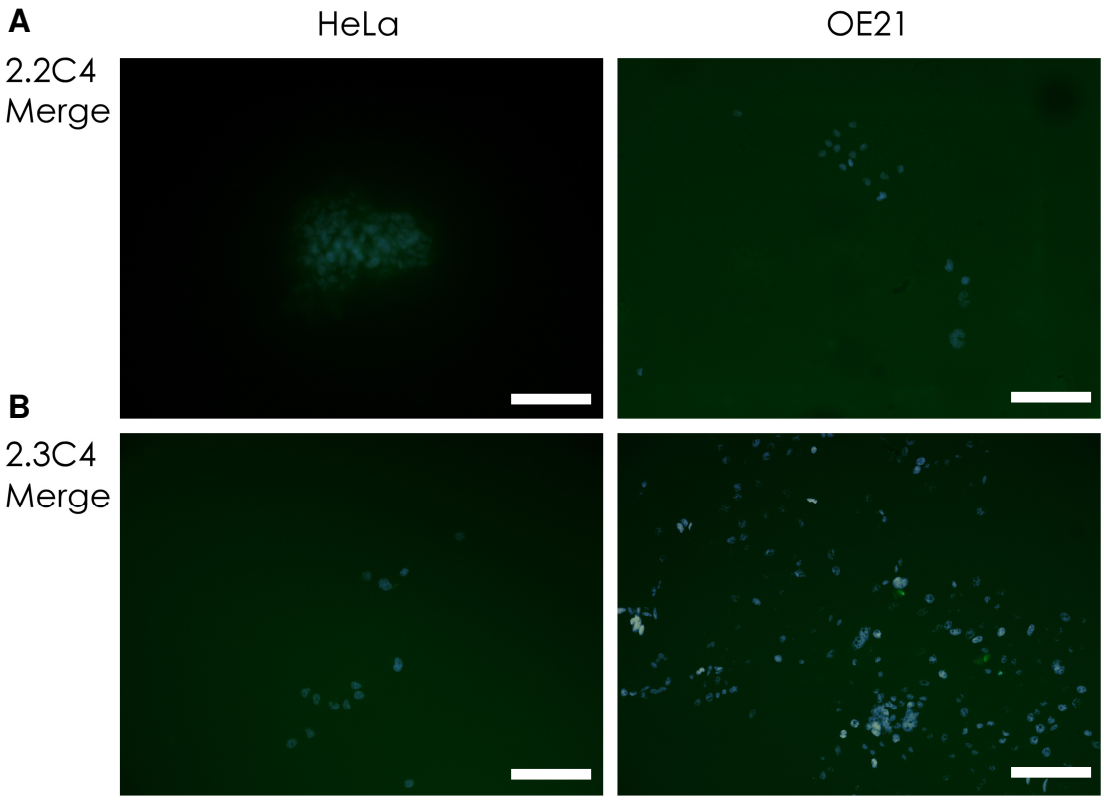

Figure 5.21: Immunofluorescence signal of PDI in HeLa and OE21

A: Untreated HeLa and B: OE21 cells were fixed and permeabilised and probed using 2.2C4 and 2.3C4. Merged DAPI/and primary supernatant data is shown. No perinuclear staining is seen, unlike with immunofluorescence for ER tracker and PDI (Figures 5.19 and 5.20). Antibody 2.3C4 was not earmarked as a candidate for Ero1α specificity, and functions here as a negative control for Ero1α. It gaves a high degree of background, and no ER localisation. Scale bars 100 μm.

5.3 Discussion

Following the generation of 61 potential monoclonal supernatants and initial Western blot testing of each one, 14 were re-tested (Table 5.1), 10 of which were potentially useful monoclonals. Both the murine and rat forms of Ero1α and Ero1β share 87-88% sequence identity. It is possible that some antibodies generated could be species specific, but this should be detected by screening against human cell lines and mouse tissues. In re-testing the antibodies, OE33 lysate was used as a positive for Ero1α, and the Ero1β immunogen was used for Ero1β. OE33 was shown to express abundant Ero1α in Chapter 3.

None of the Ero1β monoclonals showed specificity toward the protein immunogen when re-tested. This was surprising, given that the immunogen was administered as a mixture of reduced and non-reduced proteins, and that similar protein preparations (e.g an Ero1α-MBP fusion protein) have successfully produced a polyclonal antiserum to Ero1α (D5). However, the patterns obtained on non-reducing gel with some of the supernatants suggest that some further exploration could be warranted. For example, immunoprecipitation and mass spectrometry experiments could be carried out to determine the identity of the protein(s) recognised by some of the supernatants. Furthermore, it may be that the correct folding of Ero1β requires the presence of its four glycans, which would not be added by the origami bacteria used to express it. The presence of these large bulky glycans could prevent putative thermodynamically stable disulphide bonds from forming within the protein that would act to render it non-functional, and alter the final 3D structure.

One monoclonal, 2.2C4, showed some promise as a potential Ero1α antibody, and was re-tested (Figure 5.7B, Figure 5.21). However, no positive signal could be seen in the Ero1α transfectants. The monoclonal 2.2C4 was also tested in IF and compared to PDI,

a protein which shows ER localisation during immunofluorescence (Figure 5.21). Although both PDI and the ER tracker show ER localisation (Figure 5.19-Figure 5.20), 2.2C4 shows a dull fluorescence signal, and is unlikely to be ER specific. However, it could also mean that this monoclonal is not suitable for immunofluorescence microscopy.

Though the screening strategy used was sound, further work on the candidate antibodies identified is needed to evaluate them fully. In addition, the production of additional Ero1α and Ero1β mutant proteins to create proteins of a specific oxidation state could be used in future immunisation experiments. The aim remains to produce redox-state specific antibodies to analyse the functional cycle of Ero proteins in the gastrointestinal tract, and in diseases such as diabetes, or where Ero1β deficiency leads to problems with insulin folding in Ero1β -/- mice (Zito *et al.*, 2010).