**Supplementary File 1a.** Kinetic parameters for GSK3β reactions with pS45-β-catenin, related to Figures 2-3.a

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Enzyme** | **Reaction** | *k*cat (s-1) | *K*M (µM) | *k*cat/*K*M (M-1s-1) |
| pS9-GSK3β | –Axin | n.d. | n.d. (≥ 2 μM) | (4.6 ± 0.1) × 103 |
|  | +full length Axin | (2.9 ± 0.3) × 10-2 | 0.29 ± 0.08 | (1.1 ± 0.3) × 105 |
| GSK3β | –Axin | (2.8 ± 0.2) × 10-1 | 0.33 ± 0.08 | (8.5 ± 2.1) × 105 |
|  | +full length Axin | (3.4 ± 0.1) × 10-1 | 0.17 ± 0.02 | (2.0 ± 0.2) × 106 |
| GSK3β\_S9A | –Axin | (2.8 ± 0.2) × 10-1 | 0.27 ± 0.05 | (9.5 ± 1.8) × 105 |
|  | +full length Axin | (3.3 ± 0.1) × 10-1 | 0.16 ± 0.02 | (2.1 ± 0.3) × 106 |

**Supplementary File 1b.** Kinetic parameters for PKA reactions, related to Figure 4.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Substrate** | **Reaction** | *k*cat (s-1) | *K*M (µM) | *k*cat/*K*M (M-1s-1) |
| GSK3β | –Axin | (7.5 ± 0.3) × 10-3 | 0.062 ± 0.007 | (1.2 ± 0.1) × 105 |
|  | +full length Axin | (4.4 ± 0.1) × 10-3 | 0.25 ± 0.02 | (1.7 ± 0.1) × 104 |
|  | +miniAxin | (4.8 ± 0.4) × 10-3 | 0.23 ± 0.05 | (2.1 ± 0.5) × 104 |
|  | +Axin peptide | (6.4 ± 0.5) × 10-3 | 0.42 ± 0.06 | (1.5 ± 0.3) × 104 |
| CREB127-135 | –Axin | (2.3 ± 0.04) × 10-2 | 0.15 ± 0.01 | (1.5 ± 0.1) × 105 |
|  | +full length Axin | (2.3 ± 0.05) × 10-2 | 0.14 ± 0.01 | (1.6 ± 0.1) × 105 |

**Supplementary File 1c.** *K*M, ATP values for all reactions, related to Figure S3.

|  |  |  |  |
| --- | --- | --- | --- |
| **Enzyme** | **Substrate** | **Reaction** | *K*M, ATP (µM) |
| GSK3β | pS45-β-catenin | –Axin | 5.6 ± 0.9 |
|  |  | +full length Axin | 3.7 ± 1.3 |
| PKA | GSK3β | –Axin | 3.0 ± 0.6 |
|  |  | +full length Axin | 3.3 ± 0.8 |
| PKA | CREB127-135 | –Axin | 2.1 ± 0.4 |
|  |  | +full length Axin | 2.1 ± 0.2 |

**Supplementary File 1d.** Kinetic parameters for pS45-β-catenin reactions with non-PKA treated GSK3β\_S9A with and without λPPase treatment, related to Figure S12.a

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Enzyme** | **Reaction** | *k*cat (s-1) | *K*M (µM) | *k*cat/*K*M (M-1s-1) |
| GSK3β\_S9A | Not treated | (5.7 ± 0.05) × 10-2 | 0.37 ± 0.1 | (1.5 ± 0.5) × 105 |
|  | λPPase-treated | (3.2 ± 0.2) × 10-1 | 0.32 ± 0.1 | (9.9 ± 2.2) × 105 |

**Supplementary File 1e.** Values of *k*cat/*K*M for untreated, λPPase-treated, and PKA-treated GSK3β and GSK3β\_S9A in reactions with the substrate pS45-β-catenin, related to Figure S12.a

|  |  |  |
| --- | --- | --- |
| **Enzyme** | **Reaction** | *k*cat/*K*M (M-1s-1) |
| GSK3β | Untreated | (1.8 ± 0.2) × 105 |
|  | λPPase-treated | (8.5 ± 2.1) × 105 |
|  | PKA-treated | (4.9 ± 0.1) × 103 |
| GSK3β\_S9A | Untreated | (1.5 ± 0.5) × 105 |
|  | λPPase-treated | (9.9 ± 2.2) × 105 |
|  | PKA-treated | (9.5 ± 1.8) × 105 |

**Supplementary File 1f.** Protein expression plasmids, related to Methods

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Plasmid** | **Protein**a | **Expressed Protein** | **Vector**b | **Source** |
| pMG024c | GSK3β  Lambda phosphatase (λPPase) | MBP-GSK3β-HA-His  GST\_λPPase | pMBP-MG | *This study* |
| pMG071c | GSK3β\_S9A  Lambda phosphatase (λPPase) | MBP-GSK3β\_S9A-HA-His  GST\_λPPase | pMBP-MG | *This study* |
| pES001 | GSK3β | MBP-GSK3β-HA-His | pMBP-MG | *(Gavagan et al., 2020)* |
| pES002 | GSK3β\_S9A | MBP-GSK3β\_S9A-HA-His | pMBP-MG | *This study* |
|  | | | | |
| pEF073 | Axin | MBP-Axin-His | pMBP-MG | *(Gavagan et al., 2020)* |
| pMG023 | Axin384-518 (miniAxin) | MBP-Axin384-518-His | pMBP-MG | *(Gavagan et al., 2020)* |
|  | | | | |
| pMG051d | β-catenin CK1α | MBP-β-catenin-His GST-CK1α | pMBP-MG | *(Gavagan et al., 2020)* |
| pEF086 | CREB (127-135) | MBP-CREB127-135-His | pMBP-MG | *(Gavagan et al., 2020)* |
|  | | | | |
| pMG026 | Lambda phosphatase (λPPase) | His-λPPase | pBH4 | *This study* |
|  | | | | |
| H6-rC | PKA catalytic subunit | His-PKA-rC | pET15b | Addgene #14921 |
|  | | | | |

**Supplementary File 1g.** Plasmids for cell culture experiments, related to Methods

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Plasmid** | **Protein** | **Expressed Protein** | **Vector** | **Source** |
| pES1028 | Axin | Axin-mCherry | pCDNA3.1(+) | *This study* |
| pEK102 | mCherry | mCherry | pCDNA3.1(+) | *This study* |

**Supplementary File 1h.** Antibodies, related to Methods

|  |  |  |
| --- | --- | --- |
| **Antibody** | **Source** | **Identifier** |
| Anti-GSK-3β (pY216) | BD Biosciences | Cat# 612312; RRID:AB\_399627 |
| Anti-GSK-3β (pS9) | Cell Signaling Technology | Cat# 5558; RRID:AB\_10013750 |
| Anti-GSK-3β | Cell Signaling Technology | Cat# 9832; RRID:AB\_10839406 |
| MBP Tag (8G1) | Cell Signaling Technology | Cat# 2396; RRID:AB\_2140060 |
| anti-Phospho-β-Catenin (Ser33/37/Thr41) | Cell Signaling Technology | Cat# 9561; RRID:AB\_331729 |
| anti-Phospho-CREB (Ser133) | Cell Signaling Technology | Cat# 9198; RRID:AB\_2561044 |
| IRDye 800CW Donkey Anti-Mouse IgG | Li-Cor | Cat# #926-32212; RRID:AB\_621847 |
| IRDye 800CW Goat Anti-Rabbit IgG | Li-Cor | Cat# 926-32211; RRID:AB\_621843 |
| IRDye 680RD Donkey Anti-Mouse IgG | Li-Cor | Cat# 926-68072; RRID:AB\_10953628 |